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Serine mutations that abrogate ligand-induced ubiquitination and internalization of the EGF receptor do not affect c-Cbl association with the receptor

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In the present study, we examined EGF-induced internalization, degradation and trafficking of the epidermal growth factor receptor (EGFR) mutated at serines 1046, 1047, 1057 and 1142 located in its cytoplasmic carboxy-terminal region. We found the serine-mutated EGFR to be inhibited in EGF-induced internalization and degradation in NIH3T3 cells. We therefore tested the hypothesis that these mutations affect ligand-induced c-Cbl association with the receptor, leading to inhibited receptor ubiquitination. EGF was unable to induce ubiquitination of the serine-mutated EGFR, yet EGF-induced phosphorylation of the c-Cbl-binding site at tyrosine 1045, and c-Cbl-EGFR association, was unaffected. To compare the relevance of these serine residues with tyrosine 1045 in their regulation of c-Cbl binding and receptor ubiquitination, we analysed an EGFR mutated at tyrosine 1045 (Y1045F). EGF-induced c-Cbl-EGFR binding was partially inhibited, and receptor ubiquitination was abrogated in cells expressing Y1045F-EGFR. In contrast, ligand-induced internalization and degradation of the Y1045F mutant was similar to that of wild-type EGFR. Together, our data indicate that the serine residues and tyrosine 1045 are essential for EGF-induced receptor ubiquitination, but only the serine residues are critical for EGFR internalization and degradation.
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Introduction

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein with an extracellular ligand-binding domain and a cytoplasmic domain with intrinsic tyrosine kinase activity. The binding of EGF causes an increase in receptor tyrosine kinase activity by an allosteric mechanism that involves receptor dimerization (Yarden and Schlessinger, 1987; Kashles *et al*., 1988; Schlessinger, 2002). The intracellular region of the EGFR includes a regulatory carboxy-terminal domain that contains multiple autophosphorylation sites (Carpenter, 2000). Ligand-induced desensitization mechanisms are important for the regulation of transmembrane receptors like the EGFR, since its oncogenic action is related to its overexpression at the cell surface (Eccles *et al*., 1994; Franklin *et al*., 2002). Thus, the removal of activated receptor from the plasma membrane by sorting for degradation inhibits its oncogenic potential. The effects of receptor endocytosis on signaling specificity and potency remain a matter of controversy (Clague and Urbe, 2001). Receptor down-regulation was earlier described simply as signal attenuation by degradation in lysosomes. However, evidence has now accumulated that internalized receptor tyrosine kinases continue to signal from the endosomal membrane system (Di Guglielmo *et al*., 1994; Haugh *et al*., 1999).

Receptors activated by EGF are internalized via clathrin-coated pits to endosomes, and transported to lysosomes for degradation. The complex regulation of this process has been extensively studied, but the precise mechanism of how EGFR internalization and degradation is regulated is still under debate (Carpenter, 2000). It has been suggested that the activity of the receptor tyrosine kinase is essential for ligand-dependent endocytosis of the EGFR (Wiley *et al*., 1991; Lamaze and Schmid, 1995). However, it has been reported that receptor mutants with defects in their kinase activity were internalized to the same extent as wild-type receptors (Felder *et al*., 1990). The major autophosphorylation sites (tyrosines 992, 1068, 1086, 1148 and 1173) in the carboxy-terminal region of the EGFR have been shown to be essential for rapid internalization of the receptor (Helin and Beguinot, 1991; Sorkin *et al*., 1992). To add to the complexity of the regulation of EGFR internalization, phosphorylation of the EGFR at threonine 654 by PKC inhibits ligand-induced

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internalization and downregulation (Lund *et al*., 1990; Bao *et al*., 2000).

Phosphorylation of EGFR on serine and threonine residues is thought to represent a mechanism for attenuation of the receptor kinase activity. It has previously been reported that the serine 1046/1047 phosphorylation sites act to suppress signal transduction by the wild-type EGFR (Countaway *et al*., 1990; Theroux *et al*., 1992). Serines 1046, 1047, 1057 and 1142 in the carboxy-terminal region of the receptor are sites phosphorylated by the Ca^{2+}/calmodulin-dependent kinase II (CaM kinase II) (Feinmesser *et al*., 1999). Mutations of serines 1046/1047 induce fibroblast transformation and upregulate tyrosine autokinase activity that is potentiated by additional mutations of serines 1057 and 1142 (Feinmesser *et al*., 1999). Feinmesser *et al*. (1999) postulated that the mechanism for controlling EGFR tyrosine autophosphorylation may involve inhibition of cytoplasmic tail interactions with the receptor kinase domain, thereby preventing an enzyme–substrate interaction.

Of the four ErbB family members, EGFR and ErbB2 have the property of binding to the negative regulator c-Cbl (Levkowitz *et al*., 1998, 2000). Upon ligand-binding, the EGFR becomes ubiquitinated (Galcheva-Gargova *et al*., 1995). The ubiquitination process has been shown to be important for the degradation of ligand-activated EGFR and this process is directed by c-Cbl, which functions as an E3 ubiquitin ligase (Levkowitz *et al*., 1998, 1999; Joazeiro *et al*., 1999; Waterman *et al*., 1999; reviewed by Thien and Langdon, 2001). Cbl targets phosphorylated tyrosine 1045 on the activated EGFR via its amino-terminal tyrosine kinase binding (TKB) domain (Levkowitz *et al*., 1999). This interaction leads to tyrosine phosphorylation of c-Cbl and the activation of its function as a ubiquitin ligase. A mutant EGFR, whose tyrosine 1045 was changed to phenylalanine (Y1045F), has been shown to be partly defective in ubiquitination and endocytosis (Levkowitz *et al*., 1999). The Y1045F-EGFR mutant still retains a residual ability to undergo EGF-induced ubiquitination, especially in the presence of overexpressed c-Cbl and the adaptor protein Grb2 (Waterman *et al*., 2002). Therefore, it has been suggested that c-Cbl is in a complex with Grb2, and that this complex engages with EGFR via different tyrosine autophosphorylation sites (Waterman *et al*., 2002). Recently, it was reported that the Cbl-interacting protein of 85 K (CIN85) functions with c-Cbl in the regulation of ligand-induced downregulation of EGFR (Soubeyran *et al*., 2002). Upon ligand-binding c-Cbl, CIN85 and a regulatory component of clathrin-coated pits (endophilin) form a complex with the receptor, which is essential for internalization of EGFR. In this way c-Cbl uses a mechanism that is functionally different from the well characterized ubiquitin ligase activity of c-Cbl.

Here, we provide evidence that serine residues and tyrosine 1045 in the cytoplasmic carboxy-terminal domain of the EGFR are essential for EGF-induced ubiquitination. However, EGF-induced internalization was only inhibited in EGFRs with mutated serines (i.e. S1046A/S1047A and S1046A/S1047A/S1057A/S1142A) but not with the Y1045F mutation. The observed inhibition of ubiquitination and internalization of the serine-mutated EGFRs could not be explained by an inhibition of c-Cbl binding since the efficiency of binding to the serine mutants was found to be comparable to that of wild-type EGFR. Furthermore, ligand-induced degradation of serine-mutated EGFRs was disrupted, whereas Y1045F-EGFR displayed a degradation rate similar to that of the wild-type EGFR.

Results

EGF-induced internalization and degradation of EGFR require serine residues in the carboxy-terminal domain

We compared EGF-induced internalization and degradation in cells expressing wild-type EGFR and EGFR with alanine substitutions of four serine residues in the cytoplasmic carboxy-terminal domain. The serine residues at 1046, 1047, 1057 and 1142 are closely localized to tyrosine 1045, the major c-Cbl-binding site, and the five major autophosphorylation sites of the EGFR (see Figure 1 for an overview). Stable expression in NIH3T3 cells of two EGFR serine mutants (serine double mutant; DM, and serine quadruple mutant; QM, where serines 1046/1047 and serines 1046/1047/1057/1142 are mutated to alanine, respectively) was confirmed by Western immunoblotting. Mutated EGFR was expressed at equivalent levels as stably transfected wild-type EGFR (see Figure 3a). In cells stimulated with EGF for 10 min, we observed by immunofluorescence confocal microcopy decreased vesicular staining for EGFR in cells expressing serine-mutated EGFRs (Figure 2e and h) compared to wild-type EGFR (Figure 2b). Most notably the serine QM-EGFR, and to a lesser extent the serine DM, showed a pronounced plasma membrane distribution after EGF stimulation

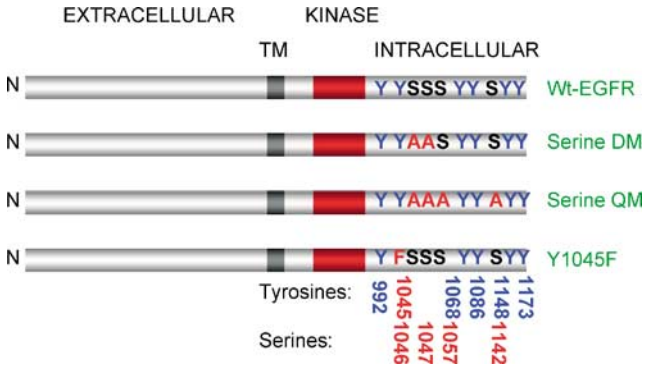

Figure 1 Schematic representation of EGFR mutants applied in the present study. Wt-EGFR, wild-type receptor; Serine DM, serine double mutant where serines 1046/1047 are changed to alanine; Serine QM, serine quadruple mutant where serines 1046/1047/1057/1142 are mutated to alanine; Y1045F, tyrosine 1045 substituted with phenylalanine. The additional major autophosphorylation sites of EGFR are indicated in blue. TM, transmembrane region

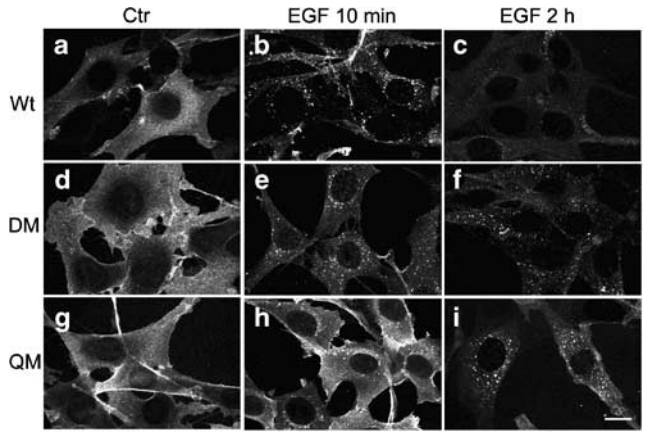

Figure 2 Immunolocalization of stably transfected wild-type EGFR (**a**–**c**) and serine DM (**d**–**f**) and QM mutants (**g**–**i**) in NIH3T3 cells. Cells were either left untreated (**a**, **d**, **g**), or exposed to EOF (10 nM) for 10 min (**b**, **e**, **h**) and 120 min (**c**, **f**, **i**). A mouse anti-EGFR directed against the extracellular region of the receptor, followed by a Alexa Fluor 546-conjugated goat anti-mouse IgG antibody were applied for immunodetection. Bar, 10 μm

(Figure 2h and e, respectively), similar to unstimulated cells (Figure 2d and g). In order to compare the longer term fate of wild-type EGFR and serine-mutated receptors following EGF stimulation, we incubated the cells with EGF on ice and chased them for 2 h at 37°C. By immunofluorescence microscopy, we observed a clear decrease in staining for wild-type EGFR after 2 h incubation with EGF (Figure 2c), whereas the decrease in receptor signal was not as profound in cells expressing serine-mutated EGFRs (Figure 2f and i). This effect was most prominent for the serine QM-EGFR, where staining with an anti-EGFR antibody revealed a heavy vesicular distribution (Figure 2i). This suggested the inhibition of ligand-induced degradation of EGFR.

To further examine these results, we analysed cell lysates by Western immunoblotting for total levels of EGFR as well as the extent of tyrosine phosphorylation after stimulation with EGF (Figure 3). In cells expressing wild-type EGFR, we observed a strong ligand-induced degradation of EGFR after 2 h incubation with EGF (Figure 3a). The EGF-induced degradation of receptors was significantly inhibited in cells expressing serine DM- and QM-EGFR when compared to wild-type EGFR. Quantitative analysis by densitometry of anti-EGFR Western blots as presented in Figure 3a showed an almost 50% inhibition of EGFR degradation for the serine QM when compared to wild-type EGFR (Figure 3b). Furthermore, Western blotting for phosphorylated tyrosine revealed that the tyrosine phosphorylated fractions of the receptor was markedly more intact in cells expressing the serine QM-EGFR compared to the serine DM and wild-type receptor (Figure 3c). Our results indicate that serine residues 1046, 1047, 1057 and 1142 are required for effective ligand-induced internalization and degradation of the EGFR.

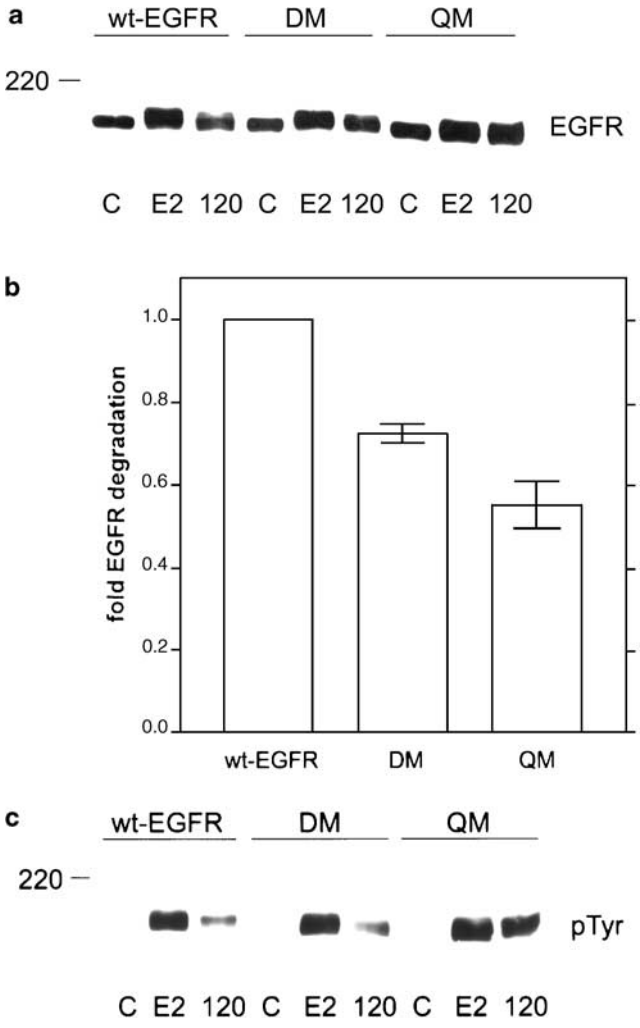

Figure 3 Western immunoblotting analysis of EGF-induced receptor degradation in wild-type EGFR and serine DM- and QM-EGFR. NIH3T3 cells stably expressing wt-receptor or serine mutants were either left untreated (C) or stimulated with EGF (10 nM) for 15 min on ice followed by chase for 2 min (E2) or 120 min at 37°C. Cell lysates were subjected to Western immunoblotting. The total level of EGFR was analysed by immunoblotting with a sheep anti-EGFR antibody (**a** and **b**). (**b**) Quantitative analysis of ligand-induced degradation of wild-type EGFR and serine-mutated receptors. Exposed films as shown in (**a**) were scanned by imaging densitometry. The mean ligand-induced degradation of EGFR after 120 min stimulation with EGF was calculated by using cells exposed to EGF for 2 min as a reference. Error bars represent s.d. (three independent experiments). (**c**) The level of tyrosine phosphorylation was analysed by immunoblotting total cell lysates from untreated and EGF-stimulated wt- and serine-mutant expressing cells with an antibody to phosphorylated tyrosine (4G10). The results in (**a**) and (**b**) are from the same experiment. Molecular weight markers indicate 220 kDa

Mutations in carboxy-terminal serine residues abolish EGFR ubiquitination without affecting c-Cbl association with the receptor

To establish whether the carboxy-terminal serine residues are required for EGFR ubiquitination, we analysed EGF-induced ubiquitination and c-Cbl–EGFR

complex formation in cells expressing wild-type EGFR and serine-mutated receptors. Total cell lysates from unstimulated cells and cells stimulated with EGF for 2 min were examined by Western immunoblotting with an antibody to EGFR and phosphorylated tyrosine (pTyr). The EGF-induced mobility shift of wild-type EGFR was readily visualized by a higher molecular weight smear, whereas cells expressing serine DM- and QM-EGFR did not display this characteristic (Figure 4a). The detection of EGF-induced smearing shown in Figure 4a is indicative of receptor ubiquitination, thus implying that this modification is abrogated in receptors mutated in carboxy-terminal serine residues. To verify this, we analysed receptor ubiquitination more directly. Cell lysates were subjected to immunoprecipitation with a mouse anti-EGFR antibody and analysed for the degree of ubiquitination by immunoblotting with an anti-ubiquitin antibody. A high molecular weight signal corresponding to ubiquitinated EGFR was detected for EGF-stimulated wild-type receptor, whereas the DM- and QM-EGFR displayed a markedly reduced level of ubiquitination (Figure 4b). Indeed, we were unable to detect ubiquitinated QM-EGFR. These results suggest that serine residues located in the carboxy-terminal region of the EGFR play an important role in the regulation of ligand-induced ubiquitination of the receptor.

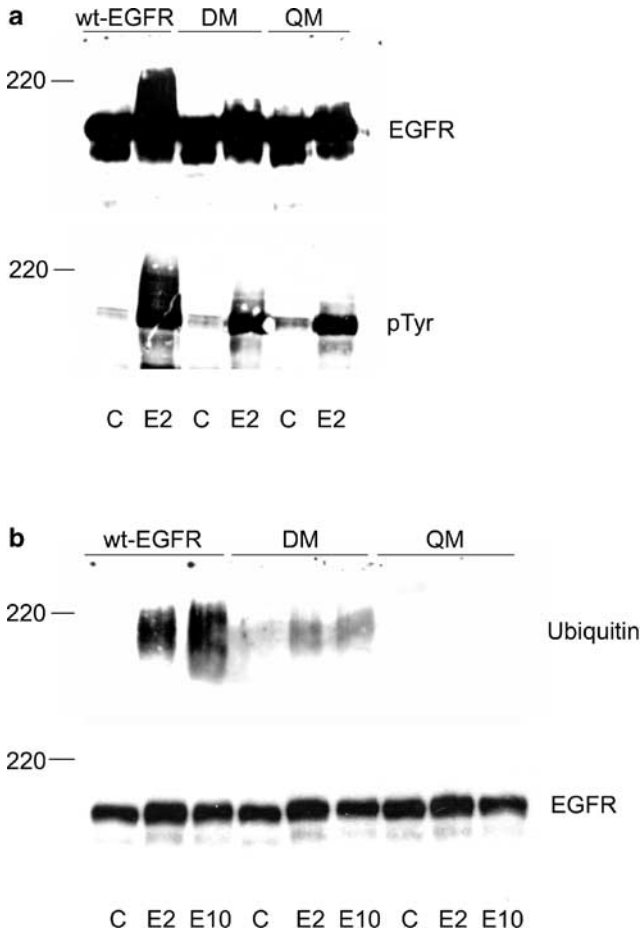

Figure 4 Analysis of ligand-induced ubiquitination of wild-type EGFR and serine DM- and QM-EGFR. NIH3T3 cells expressing wt receptor or serine mutants were either left untreated (C) or exposed to 10 nM EGF for 2 or 10 min (E2 and E10, respectively). (**a**) Total cell lysates were analysed by Western immunoblotting with a sheep anti-EGFR antibody (upper panel) or an antibody to phosphorylated tyrosine (4G10; lower panel). (**b**) Cell lysates were subjected to immunoprecipitation with a sheep anti-EGFR antibody and the coprecipitates were analysed by Western blotting with a mouse antiubiquitin antibody. The molecular weight markers at left indicate −220 kDa

It is of significant interest that the mutated serine residues at 1046 and 1047 are immediately adjacent to tyrosine 1045, the major binding site for the ubiquitin ligase c-Cbl. Since ubiquitination of the EGFR is linked to c-Cbl binding, we tested whether mutations in serines affect phosphorylation of tyrosine 1045 and c-Cbl–EGFR complex formation. We analysed EGFR immunoprecipitates for EGF-induced phosphorylation of tyrosine 1045 by immunoblotting with an antibody specific to phosphorylated tyrosine 1045. Both the serine DM- and QM-EGFR exhibited ligand-induced phosphorylation of tyrosine 1045 equivalent to that of wild-type EGFR (Figure 5a). This suggested that the substrate specificity of the kinase (i.e. the EGFR or a Src kinase) is not dependent on the retention of serine residues at positions +1 and +2 relative to Y1045. To examine whether mutations of the specified serines affected the receptor's affinity for c-Cbl, we analysed immunoprecipitated c-Cbl for its ability to coprecipitate the EGFR. Immunoprecipitates were first Western-blotted with an anti-Cbl antibody, and then reblotted with anti-EGFR. Immunoprecipitates from cells treated with EGF for 2 and 10 min demonstrated a complex formation between EGFR and c-Cbl that was not affected by mutations of carboxy-terminal serines residues in the receptor (Figure 5b). To confirm these results, total cell lysates and anti-EGFR immunoprecipitates from unstimulated and EGF-stimulated cells were immobilized onto nitrocellulose membranes and probed with a GST-v-Cbl fusion protein, as described previously (Thien and Langdon, 1997a). The v-Cbl protein is an oncogenic variant of c-Cbl with a large truncation that deletes all sequences except the amino-terminal TKB domain. Subsequent immunoblotting with an anti-GST antibody detected GST-v-Cbl bound to the immobilized EGFR from EGF-stimulated but not unstimulated cells (Figure 5c). Significantly, this interaction was not inhibited in cells expressing receptors with mutations in the carboxy-terminal serines. Our results demonstrate that although mutations in carboxy-terminal serines abolish EGFR ubiquitination, the phosphorylation of tyrosine 1045 and the binding of c-Cbl to the receptor are not affected.

Mutant Y1045F-EGFR displays inhibited ligand-induced c-Cbl binding and ubiquitination without affecting receptor internalization

In view of our results with EGFRs mutated in carboxy-terminal serines, and the close proximity of three of

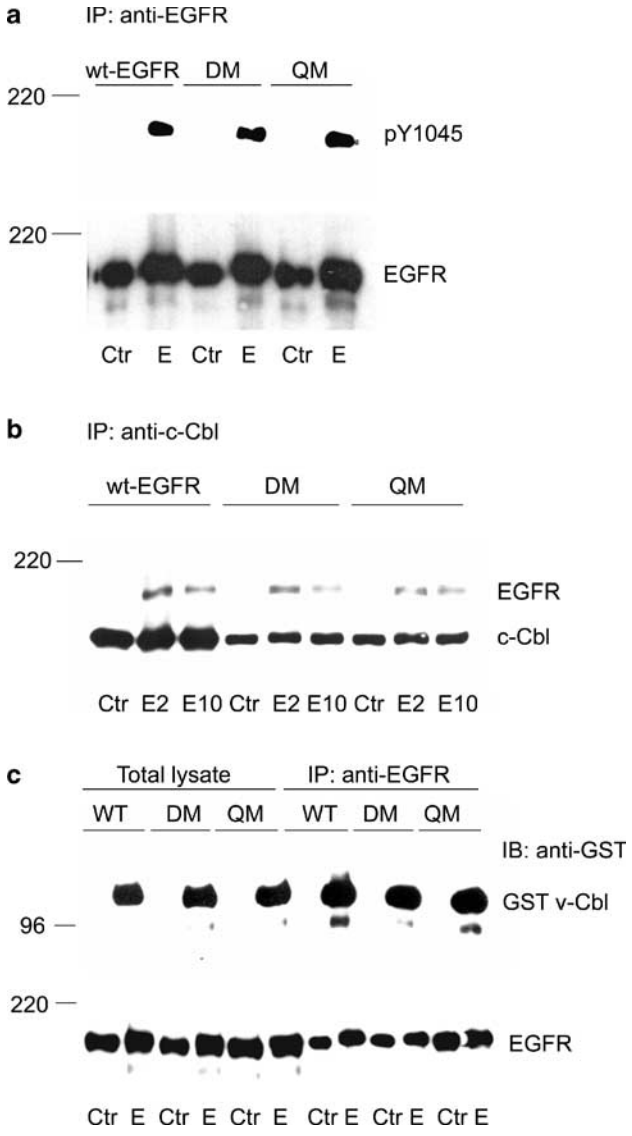

Figure 5 Comparison of EGF-induced c-Cbl binding to wild-type EGFR and serine DM- and QM-EGFR. (**a**) Cells were either left untreated (Ctr) or stimulated with 10 nM EGF for 2 min (E) and total cell lysates were prepared. Cell lysates were subjected to immunoprecipitation with a sheep anti-EGFR antibody and the bound proteins were resolved by SDS–PAGE and analysed by Western blotting with a rabbit antiphosphorylated Y1045 antibody (upper panel) or a sheep anti-EGFR antibody to demonstrate equivalent protein levels (lower panel). Molecular weight markers at left indicate 220 kDa. (**b**) Lysates from unstimulated cells (Ctr) or cells exposed to 10 nM EGF for 2 or 10 min (E2 and E10, respectively) were subjected to immunoprecipitation with a rabbit anti-c-Cbl antibody. The coprecipitates were analysed by Western blotting with a mouse anti-c-Cbl antibody, and the membrane was reprobed with a sheep anti-EGFR antibody. The molecular weight marker at left indicates 220 kDa. (**c**) Lysates from unstimulated cells (Ctr) or cells exposed to 10 nM EGF for 2 min (E) were either analysed directly (total lysate) or subjected to immunoprecipitation with a sheep anti-EGFR antibody. Total lysates and coprecipitates were resolved by SDS–PAGE and probed with a GST-v-Cbl fusion protein (upper panel). Bound fusion protein was detected by blotting with a mouse anti-GST antibody. Western blotting with a sheep anti-EGFR antibody was performed to demonstrate equivalent protein levels (lower panel). Molecular weight markers at left indicate 96 kDa (upper panel) and 220 kDa (lower panel)

these residues to tyrosine 1045, we constructed a Y1045F-EGFR mutant. Phenylalanine substitution (Y1045F) of this site has previously been shown to reduce ligand-induced receptor ubiquitination and downregulation in CHO cells (Levkowitz *et al*., 1999). For an initial characterization, we prepared total lysates from cells expressing wild-type EGFR and Y1045F-EGFR, and these were analysed for ligand-induced phosphorylation of Y1045. Western blotting confirmed that EGF-induced phosphorylation of tyrosine 1045 was abrogated in cells expressing Y1045F-EGFR (Figure 6a). In contrast, EGF-induced phosphorylation of the major autophosphorylation site at tyrosine 1173 in the EGFR was not inhibited in the Y1045F-EGFR mutant. Next, we analysed c-Cbl immunoprecipitates by immunoblotting with an antibody specific to phosphotyrosine 1173 of the EGFR. We observed an EGF-dependent complex formation between phosphorylated EGFR and c-Cbl, which was not noticeably inhibited in cells expressing the Y1045F-EGFR mutant (Figure 6b). To further investigate the impact of mutating tyrosine 1045 on ligand-induced ubiquitination of the EGFR, total cell lysates were prepared from unstimulated and EGF-treated cells expressing wild-type EGFR and the Y1045F mutant, and analysed by Western immunoblotting with an anti-EGFR antibody. Stimulation with EGF for 2 min led to a high molecular weight smearing of the wild-type EGFR, but not for the Y1045F-EGFR (Figure 6c; upper panel). Consistent with previous work (Levkowitz *et al*., 1999), our data indicate that mutation of tyrosine 1045 blocked ligand-induced ubiquitination of the EGFR. The observed inhibited ubiquitination in cells expressing the Y1045F mutant was confirmed by Western blotting analysis of EGFR immunoprecipitates with an antibody to ubiquitin (Figure 6c; lower panel).

Inhibition of ligand-induced internalization of Y1045F-EGFR has recently been described (Levkowitz *et al*., 1999; Waterman *et al*., 2002). We therefore deduced that EGF-induced internalization of the Y1045F-EGFR in our cell system would be affected. We analysed the subcellular localization of Y1045F-EGFR by confocal immunofluorescence microscopy. Unstimulated control cells and cells stimulated with EGF for 10 min were studied with an antibody to EGFR. Wild-type EGFR displayed an EGF-induced redistribution from the plasma membrane to vesicles, demonstrated by a distinct punctuate staining observed in the EGF-stimulated cells (Figure 7a and b). EGF-induced internalization of Y1045F-EGFR was similar to that for wild-type EGFR, and reduced vesicular staining for the mutant was not detected (Figure 7c and d). Taken together, our results provide evidence that tyrosine 1045 is essential for receptor ubiquitination, but may not be necessary for the formation of an inducible association between c-Cbl and the receptor, as shown recently by Waterman *et al*. (2002). Furthermore, internalization of the EGFR appears to be independent of a requirement for ubiquitination and the retention of tyrosine 1045.

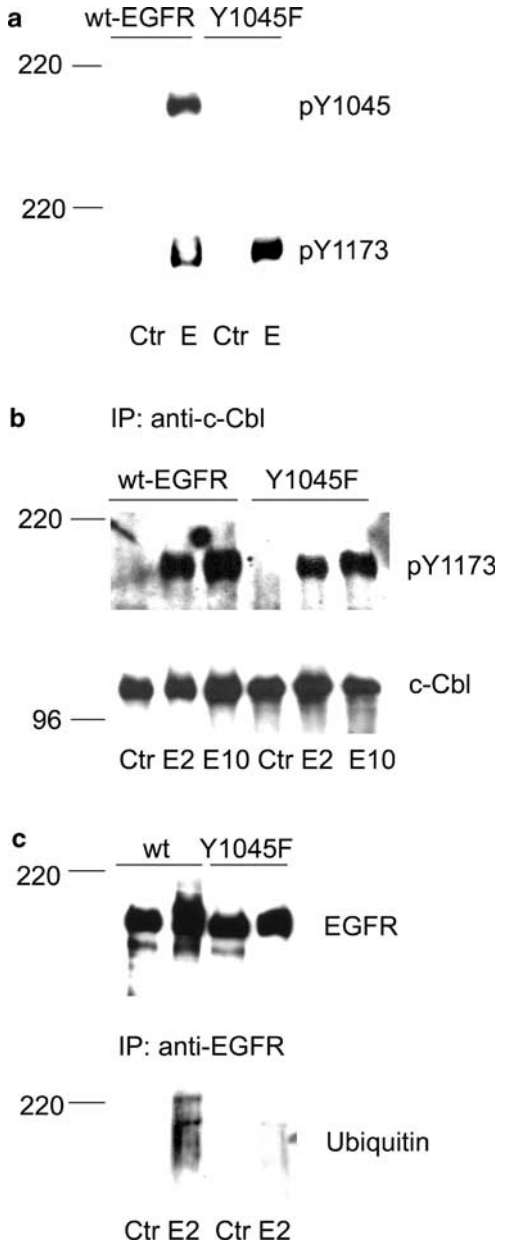

Figure 6 Comparison of EGF-induced c-Cbl–EGFR complex formation and ubiquitination in cells expressing wild-type EGFR and Y1045F-EGFR. (**a**) Lysates from unstimulated cells (C) or cells stimulated with 10 nM EGF for 2 min (E) were analysed by Western blotting with a rabbit anti-phosphorylated Y1045 (upper panel) or a rabbit anti-phosphorylated Yl173 (lower panel). (**b**) Lysates were subjected to immunoprecipitation with a rabbit anti-c-Cbl antibody, and the bound proteins were analysed by Western blotting with a rabbit anti-phosphorylated Y1173 (upper panel). A Western blot with a mouse anti-c-Cbl antibody is shown to demonstrate equivalent levels of c-Cbl in the precipitates (lower panel). (**c**) Cell lysates from unstimulated cells (C) and cell stimulated with 10 nM EGF for 2 min (E2) were prepared. The cell lysates were either analysed directly by Western blotting with a sheep anti-EGFR antibody (upper panel) or subjected to immunoprecipitation with a sheep anti-EGFR antibody and Western blotting with a mouse anti-ubiquitin antibody (lower panel). Molecular weight markers at left indicate 220 kDa

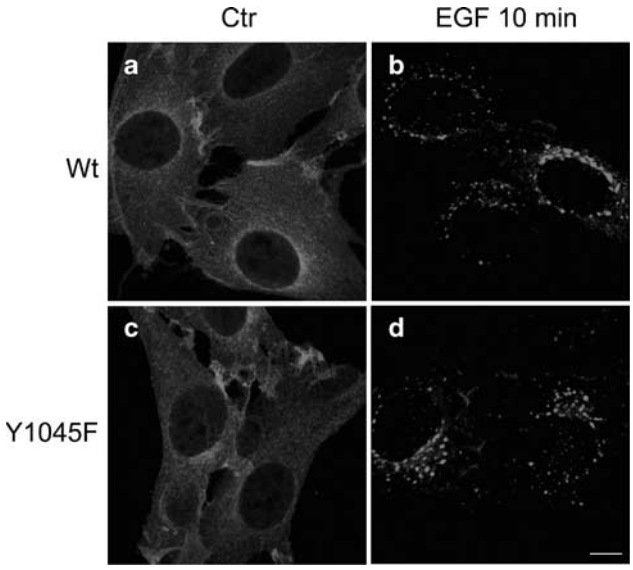

Figure 7 Immunofluorescence microscopy of ligand-induced distribution of stably transfected wild-type EGFR and Y1045F-EGFR. Cells expressing wild-type EGFR (**a** and **b**) and Y1045F-EGFR (**c** and **d**) were either left untreated (**a** and **c**) or stimulated with 10 nM EGF for 10 min (**b** and **d**). A sheep anti-EGFR antibody followed by a Cy3-conjugated donkey anti-sheep IgG antibody were applied for immunodetection. Bar, 10 μm

Tyrosine 1045 is not essential for EGF-induced degradation of EGFR

The findings described above indicate that ligand-induced receptor ubiquitination at the plasma membrane, which occurs at an early time point after stimulation, is dependent on the tyrosine 1045 site. However, inhibited ubiquitination of the Y1045F-EGFR mutant did not affect ligand-induced internalization of the receptor (Figure 7d). The possibility that ubiquitination of the receptor could be more important in the regulation of its degradation was therefore investigated. This was tested by comparing the total levels of EGFR in cells expressing wild-type EGFR and Y1045F-EGFR. The cells were either left unstimulated or exposed to EGF for 1 and 2 h. After stimulation with EGF, the total level of receptor was significantly reduced for cells expressing wild-type and Y1045F-EGFR, such that the degradation of the Y1045F mutant was equivalent to that observed for wild-type EGFR (Figure 8). This indicates that tyrosine 1045 is not essential for EGF-induced degradation of the receptor in NIH3T3 cells.

Discussion

In this study, we have demonstrated that effective internalization and degradation of EGFR in NIH3T3 cells following EGF stimulation is dependent on distinct serine residues in the carboxyl-terminal region of the

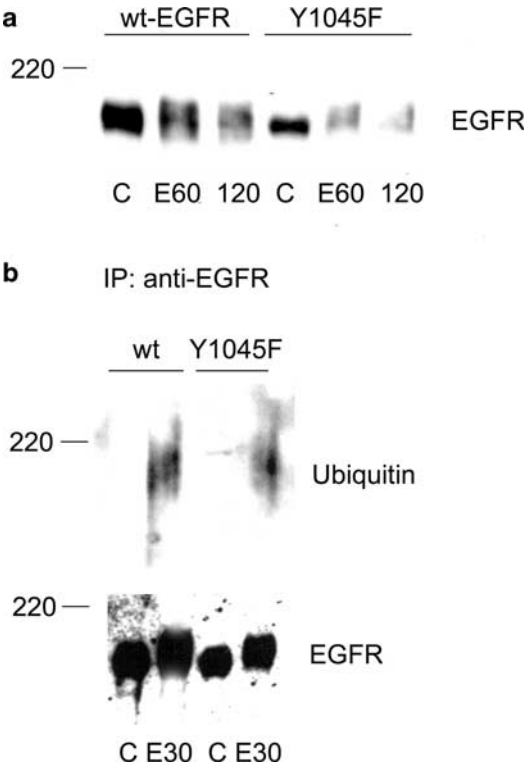

Figure 8 Comparison of ligand-induced degradation of EGFR in cells stably expressing wild-type EGFR or Y1045F-EGFR. (**a**) Lysates from unstimulated cells (C) or cells stimulated with 10 nM EGF for 60 and 120 min were analysed by Western blotting with a sheep anti-EGFR. The molecular weight marker at left indicates 220 kDa

receptor. We showed that while ubiquitination of the EGFR was abolished when these serine residues were mutated, the phosphorylation of tyrosine 1045 and c-Cbl binding to the receptor was not affected. Furthermore, we have demonstrated that cells expressing Y1045F-EGFR also display abrogated ubiquitination of the receptor at an early time point after stimulation, but this does not noticeably inhibit EGFR internalization and degradation.

Inhibited internalization of the EGFR mutated in serine residues 1046/1047 has been described earlier (Theroux *et al*., 1992). It has been proposed that the acute desensitization of EGFR function after stimulation was accounted for, in part, by the effect of EGF to increase phosphorylation of the receptor at serines 1046/1047 (Countaway *et al*., 1992). Indeed, the substitution of serines 1046/1047 was demonstrated to cause a potentiation of signal transduction by increased ligand -induced tyrosine phosphorylation (Theroux *et al*., 1992). It has been shown that serines 1046/1047 as well as serines 1057/1142 in the carboxyl-terminal EGFR tail are sites for calcium/calmodulin-dependent kinase II (CaMKII) phosphorylation (Feinmesser *et al*., 1999). It was postulated that these serine residues are important in the regulation of EGFR tyrosine autophosphorylation by promoting an interaction between the cytoplasmic tail with the kinase domain (Feinmesser *et al*., 1999). However, it has not been ruled out whether the effect on the receptor tyrosine kinase activity by mutation of these serine residues is due to inhibition of EGFR internalization. It has recently been demonstrated that calmodulin antagonists stimulate EGFR tyrosine phosphorylation and inhibit degradation of the receptor probably by modifying the structure of the endocytic compartments (Tebar *et al*., 2002). Our initial studies demonstrated inhibited ubiquitination of serine-mutated EGFRs, and therefore we hypothesized that the block we observe on receptor internalization may be a direct consequence of inhibited ubiquitination. This would be in agreement with previous findings that ubiquitination is linked to internalization of the EGFR (Levkowitz *et al*., 1999). However, residual EGF-induced downregulation of Y1045F was detected when c-Cbl was overexpressed (Levkowitz *et al*., 1999). Furthermore, as a consequence of our demonstrations that ligand-induced internalization of the Y1045F mutant is not inhibited in spite of abrogated ubiquitination, we decided to alter our hypothesis that the inhibited internalization of EGFR mutated in serines was linked to the abolished ubiquitination. Our data presented here suggest that the requirement for ubiquitination upon ligand-induced internalization of the EGFR is not absolute, and that other elements like the structure of the cytoplasmic receptor tail may be more critical. Consistent with this notion, ubiquitin-independent internalization of a truncated growth hormone receptor has been reported (Covers *et al*., 1998).

Recently, a direct role for c-Cbl in receptor-mediated endocytosis was described, which is functionally separable from its ubiquitin ligase activity (Soubeyran *et al*., 2002). It was demonstrated that c-Cbl recruits Cbl-interacting protein of 85 kDa (CIN85) and regulatory components of clathrin-coated pits (endophilin) to form a complex with ligand-activated EGFR. Disruption of this interaction is sufficient to block endocytosis of the receptor (Soubeyran *et al*., 2002). In view of these studies, an alternative explanation for an inhibition of receptor internalization is possible. Mutations in carboxy-terminal serine residues of the EGFR may cause an interruption of the interaction between the endocytosis machinery and the carboxy-terminal receptor tail containing c-Cbl-CIN85-endophilin.

There are at least three potential mechanisms that could explain the observed inhibition of EGFR ubiquitination by mutations of serines and Y1045F: (i) Receptor ubiquitination is dependent on the precise orientation between the receptor and c-Cbl and that this alignment is disrupted in EGFRs where carboxyl-terminal serines and tyrosine 1045 are mutated. (ii) It is possible that proteins in addition to c-Cbl are essential during initiation of ligand-induced EGFR ubiquitination and that they are dependent on serine residues and Y1045 for binding to the receptor. (iii) Alternatively, the inhibition of ubiquitination can be related to effects on the activity of the EGFR tyrosine kinase. EGF-induced ubiquitination of the EGFR is dependent upon EGFR kinase activity, as reported recently (Longva *et al*., 2002). It is probable that a combination of the

mechanism suggested in (i) and (iii) is involved. In this way, the regulation of EGFR tyrosine kinase activity is dependent on interactions with distinct conformations of the EGFR carboxy-terminal tail where serine and tyrosine 1045 residues are essential. It has been reported that mutations at serines 1046/1047/1057/1142 increased the receptor tyrosine autokinase activity (Feinmesser *et al*., 1999). It is known that c-Cbl regulates the activity of receptor tyrosine kinases like the EGFR (Thien and Langdon, 1997b), but an understanding of this regulation remains incomplete. Thien and Langdon (1997b) reported that expression of an oncogenic form of c-Cbl (70Z-Cbl) without a functional ubiquitin-conjugating enzyme-binding RING finger and linker domain enhances the EGFR tyrosine kinase activity. More recently, it has been described that the same mutation of c-Cbl abolishes EGF-induced internalization and downregulation of the EGFR (Thien *et al*., 2001). In unstimulated cells, we could detect a small increase in receptor tyrosine phosphorylation in cells expressing serine DM- and QM-EGFR (see Figure 4a, lower panel). Mutations of serines 1046/1047/1057/1142 can therefore have an impact on the EGFR tyrosine kinase analogous to that of the 70Z-Cbl mutant.

In our study of mutations of serines 1046/1047/1057/1142, we found that they did not have an impact on c-Cbl recruitment to the EGFR. Therefore, the observed inhibition of ubiquitination could not be explained by an inhibition of c-Cbl binding, but as pointed out above they may alter the way c-Cbl and the receptor are orientated with respect to each other. Initially, we expected mutations of neighboring residues to the major c-Cbl binding site, that is, tyrosine 1045, to have some effects on tyrosine 1045 phosphorylation and c-Cbl–EGFR complex formation. Previous studies have shown that binding of c-Cbl to tyrosine 1045 is required for ubiquitination and downregulation of the receptor (Levkowitz *et al*., 1999). We therefore initiated an analysis of the Y1045F-EGFR mutant and found that the tyrosine 1045 site is not crucial for c-Cbl binding, since the interaction was not significantly different from that observed for wild-type EGFR. Interactions of c-Cbl with the adapter protein Grb2 and coupling of the c-Cbl–Grb2 complex to EGFR have been described earlier (Meisner and Czech, 1995; Meisner *et al*., 1995). Waterman *et al*. (2002) recently reported that ubiquitination of Y1045F-EGFR is possible, especially when coexpressing c-Cbl and Grb2. Our results demonstrating that c-Cbl can interact with Y1045F-EGFR in NIH3T3 cells are consistent with these results. We observed an abrogated ligand-induced ubiquitination for the Y1045F mutant, but it is important to note that we are studying endogenous levels of c-Cbl, whereas other groups, for the most part, employ cells overexpressing c-Cbl. Taken together, our results demonstrate that mutations of either carboxyl-terminal serines or tyrosine 1045 abolish EGFR ubiquitination without disrupting c-Cbl binding to the receptor.

The involvement of serine residues in the regulation of ubiquitination has been reported earlier where a six-amino-acid motif was proposed to be involved in targeting of both β-catenin and IκBα for ubiquitination (Chen *et al*., 1995; Orford *et al*., 1997). A serine to alanine mutation within this ubiquitination targeting sequence stabilizes the protein by inhibiting its ubiquitination (Orford *et al*., 1997). β-catenin and IκBα involve ubiquitin ligases other than c-Cbl, and the engagement of additional ubiquitin ligases for EGFR ubiquitination is possible. It has recently been reported that two different classes of E3 ligases (CBLC and AEP4) can interact and cooperate to downregulate EGFR signaling in yeast (Courbard *et al*., 2002). Furthermore overexpression of c-Cbl, which has been a usual strategy for studying receptor ubiquitination, may mask the role of another ligase.

In summary, our results demonstrate that in situations where binding of functional c-Cbl to the EGFR is intact, it is still possible to abolish ubiquitination of the receptor. Mutations of carboxy-terminal serine residues or the tyrosine 1045 lead to abrogation of ligand-induced EGFR ubiquitination coincident with c-Cbl binding to the receptor. Thus, we suggest that the initiation of EGFR ubiquitination requires several different domains within the carboxy terminus that include the key serine residues described in this report and tyrosine 1045.

Materials and methods

Materials

A mouse antibody to the extracellular domain of EGFR, a rabbit anti-c-Cbl and a mouse anti-GST were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A sheep antibody to the intracellular domain of EGFR was obtained from Fitzgerald (Concord, MA, USA). The R42/pYl173 rabbit antisera are specific to phosphorylated Tyr 1173 in EGFR and has previously been described (Oksvold *et al*., 2000). The mouse anti-phosphotyrosine antibody 4G10 was kindly provided by Dr Brian Druker. Mouse anti-c-Cbl was purchased from BD Transduction Laboratories (San Diego, CA, USA). A mouse anti-ubiquitin that detects mono- and polyubiquitin was purchased from Stressgen (San Diego, CA, USA). Alexa Fluor 546-conjugated goat anti-mouse IgG was obtained from Molecular Probes (Eugene, OR, USA). Cy3-conjugated donkey anti-sheep IgG and Cy2-conjugated donkey anti-rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Peroxidase-conjugated donkey anti-mouse and anti-sheep IgG were obtained from Jackson ImmunoResearch. Electrophoresis reagents were purchased from BioRad (Hercules, CA, USA). Reagents for site-directed mutagenesis and subcloning were purchased from Promega (Madison, WI, USA) if not specified. All other reagents were obtained from Sigma Chemical Company (St Louis, MO, USA), unless otherwise stated.

Cell culture and transfection

NIH3T3 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM; Trace Scientific, Noble Park, Australia) containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA), and 2 mM L-glutamine (Trace Scientific) at 37°C and 5% CO_2. Cells were stably transfected with human full-length EGFR by retroviral infection, as described earlier (Thien and Langdon, 1997a).

The EGFR serine mutants expressed in the pLEN vector (EGFR-S1046A/S1047A and EGFR-S1046A/S1047A/S1057A/S1142A) have been described earlier (Feinmesser *et al*., 1999). The vector control and mutants were electroporated into Ψ2 packaging cells to generate virus particles for infection of NIH3T3 cells, which were selected with 400 μg/ml G418 (Gibco BRL) (Thien and Langdon, 1997a). All constructs were examined by DNA sequensation.

Site-directed mutagenesis of tyrosine 1045 in EGFR

The *Sph*I site in the pUC19-human EGFR (hEGFR) was destroyed by digestion, blunt ended with T4 DNA polymerase and religated. This construct was used as a template for PCR amplification of the EGFR C-terminal domain. Two regions were amplified by PCR using PfuTurbo polymerase (Stratagene, La Jolla, CA, USA). A *Bg*lll/*Sph*I digested product with the Y1045F mutation was constructed with the oligonucleotides 5′-GACCTTTGGATCCAAGCC-3′ (forward) and 5′-GTCTGAGCATGCTAAATCGCTGCAAGAAGC-3′ (reverse). In addition, a *Sph*I/*Sal*I digested product including the EGFR 3′ end was constructed with the oligonucleotides 5′-TTGCAGCGATTTAGCATGCTCAGACCCCACAG-3′ (forward) and 5′-AGCGGATAACAATTTCACACAGG-3′ (reverse). Triple ligation of EGFR-Y1045F-*Bgl*II/*Sph*I, EGFR-*Sph*I/*Sal*I and pUC19-EGFR(*Sph*I)-*Bgl*II/*Sal*I) was made. The pUC19/hEGFR(Y1045F + *Sph*I) was digested with *Sph*I, blunt ended with T4 DNA polymerase and religated. The pUC19/hEGFR(Y1045F) was digested with *Kpn*I, blunt ended and redigested with *Sal*I. The 4 kb hEGFR(Y1045F)-*Kpn*I/*Sal*I fragment was isolated and subcloned into pBabe-Puro-*Sna*BI/*Sal*I. For stable expression, the pBabePuro construct was electroporated into Ψ2 packaging cells to generate virus particles for infection of NIH3T3 cells, which were selected with 2 μg/ml puromycin (Thien and Langdon, 1997a). The construct was examined by DNA sequensation.

Stimulation with EGF

Cells cultured in Petri dishes or on cover slips were starved in DMEM containing 0.5%. fetal bovine serum over night, and exposed to 10 nM EGF and chased at 37°C. Cells were either stimulated by continuous exposure to EGF in DMEM, or incubated with EGF in Hanks balanced salt solution for 15 min on ice followed by washing and chasing at 37°C. Cells were rinsed in ice-cold PBS, and prepared for Western immunoblotting, immuno coprecipitation and immunofluorescence microscopy analyses.

Western immunoblotting analysis

Cells were washed in ice-cold PBS and lysed in Laemmli buffer, pH 7.4. (10 mM Tris-HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 5 mM EDTA, 2.5 μg/ml aprotinin and leupeptin, 1 mM β-glycerophosphate, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and 1 mM Na_3VC_4). Further sample preparations were performed as described elsewhere (Oksvold *et al*., 2000). The nitrocellulose membranes were incubated overnight at 4°C with primary antibodies. Peroxidase-conjugated anti-mouse and anti-rabbit IgG for 90 min at RT were used for detection. All antibodies were diluted in 1% (w/v) fat-free dry milk (Bonlac Foods, Melbourne, Australia) in TBS containing 0.01% thimerosal. The filters were washed in TBS, and antigens were visualized by the enhanced chemiluminescence (ECL) method with Hyperfilm MP (Amersham Biosciences, Little Chalfont, UK).

Immunofluorescence microscopy

Cells cultured on cover slips were washed in PBS and fixed in 4% paraformaldehyde in PBS, pH 7.4, for 15 min, and permeabilized in 0.2% Triton X-100 for 5 min. Cells were incubated with primary antibodies overnight, followed by Alexa Fluor 546-, Cy2- or Cy 3- conjugated goat or donkey antibodies to IgG of the appropriate species. All dilution of antibodies were in PBS containing 1% BSA. Cover slips were mounted with Slow Fade Light Antifade reagent (Molecular Probes). Cells were studied in a Nikon Diaphot 300 microscope equipped for laser scanning confocal microscopy (BioRad MRC 1000/1024). A $\times$60/1.4 oil objective was used.

Immuno coprecipitation

Cells were washed in ice-cold PBS and lysed in Tris-buffered saline, pH 7.4. (50 mM Tris, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 2.5 μg/ml aprotinin and leupeptin, 1 mM β-glycerophosphate and AEBSF, and 10 mM iodoacetate). Lysates were incubated on ice for 15 min before cell debris and nuclei were removed by centrifugation at 5000 *g* for 5 min. Protein G–Sepharose (Amersham Biosciences) in a 50 : 50 mixture in 50 mM Tris buffer, pH 7.0, was incubated with mouse anti-Cbl or anti-EGFR for 2 h at RT. Cell lysates were added to the washed protein G–Sepharose–antibody complex, and incubated for another 1 h on ice. The immunoprecipitates were washed 3 $\times$ in Tris-buffered saline and boiled for 5 min in 100 μl Laemmli buffer containing 0.02% bromophenolblue and 2% β-mercaptoethanol.

Filter binding of GST-v-Cbl

A GST-v-Cbl fusion protein was prepared as described earlier (Thien and Langdon, 1997a). Total cell lysates and immunoprecipitates of EGFR were separated by SDS–PAGE as described. Proteins were transferred to a nitrocellulose membrane and incubated with 5% dry milk in TBS with 1 mM Na_3VO_4 and 10 mM NaF for 30 min. The membrane was probed with 2 μg/ml GST-v-Cbl in the same solution at 4°C for 4 h prior to an 8 $\times$ 2 min wash in TBS. The membrane was incubated with anti-GST antibodies at 4°C overnight and processed as described earlier for Western immunoblotting analysis.

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