Epidermal Growth Factor Receptor Phosphorylation Sites Ser<sup>991</sup> and Tyr<sup>998</sup> Are Implicated in the Regulation of Receptor Endocytosis and Phosphorylations at Ser<sup>1039</sup> and Thr<sup>1041</sup>*

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Aberrant expression, activation, and down-regulation of the epidermal growth factor receptor (EGFR) have causal roles in many human cancers, and post-translational modifications including phosphorylation and ubiquitination and protein-protein interactions directly modulate EGFR function. Quantitative mass spectrometric analyses including selected reaction monitoring (also known as multiple reaction monitoring) were applied to the EGFR and associated proteins. In response to epidermal growth factor (EGF) stimulation of cells, phosphorylations at EGFR Ser<sup>991</sup> and Tyr<sup>998</sup> accumulated more slowly than at receptor sites involved in RAS-ERK signaling. Phosphorylation-deficient mutant receptors S991A and Y998F activated ERK in response to EGF but were impaired for receptor endocytosis. Consistent with these results, the mutant receptors retained a network of interactions with known signaling proteins including EGF-stimulated binding to the adaptor GRB2. Compared with wild type EGFR the Y998F variant had diminished EGF-stimulated interaction with the ubiquitin E3 ligase CBL, and the S991A variant had decreased associated ubiquitin. The endocytosis-defective mutant receptors were found to have elevated phosphorylation at positions Ser<sup>1039</sup> and Thr<sup>1041</sup>. These residues reside in a serine/threonine-rich region of the receptor previously implicated in p38 mitogen-activated protein kinase-dependent stress/cytokine-induced EGFR internalization and recycling (Zwang, Y., and Yarden, Y. (2006) p38 MAP kinase mediates stress-induced internalization of EGFR: implications for cancer chemotherapy. EMBO J. 25, 4195–4206). EGF-induced phosphorylations at Ser<sup>1039</sup> and Thr<sup>1041</sup> were blocked by treatment of cells with SB-202190, a selective inhibitor of p38. These results suggest that coordinated phosphorylation of EGFR involving sites Tyr<sup>998</sup>, Ser<sup>991</sup>, Ser<sup>1039</sup>, and Thr<sup>1041</sup> governs the trafficking of EGF receptors. This reinforces the notion that EGFR function is manifest through spatially and temporally controlled protein-protein interactions and phosphorylations.

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Production (e.g., ERK kinase activation) and others that accumulate more slowly following ligand treatment and are involved in receptor internalization and down-regulation (5–11). Although advances in MS and the definition of phosphorylation-dependent protein-protein interactions have led to a greatly expanded view of EGFR function and regulation, our understanding of the biological consequences and spatial-temporal relationships of individual modifications is incomplete.

In a previous quantitative phosphoproteomics study aimed at the identification of drug-modulated changes in phosphorylation associated with the EGFR network, a cluster of three sites of phosphorylation in the EGFR carboxyl tail region was identified as affected by receptor stimulation by EGF and inhibited by the ATP-competitive EGFR inhibitor PKI166 in human A431 tumor cells and xenograft tumors (12). The three sites in the cluster, Ser991, Ser995, and Tyr998, are localized within a single tryptic peptide having the sequence MHLP-SPTDSNFRY that spans residues 987–999. The phosphorylation of Tyr998 was first described by Stover et al. (12), whereas the two serine sites were shown previously to be phosphorylated by Heisermann and Gill (13). Numerous recent studies using different cultured cell models have verified the phosphorylation of EGFR at Tyr998 and Ser991 (10, 11, 14, 15), and Thr993 was also observed to be phosphorylated within this same region of the EGFR in EGF-stimulated HeLa cells (10). The modulation of these sites by EGF and the EGFR inhibitor implicates them in EGFR signaling and suggests that they may have utility as pharmacodynamic markers of EGFR activity. However, the function and importance of these sites, their modulation by kinases and phosphatases, and possible roles in EGFR function remain unknown.

Several amino acid residues in the EGFR have been implicated in the regulation of its trafficking. Sorkin et al. (16) showed that substitution of Tyr998 with phenylalanine rendered high density EGFRs defective for endocytosis and interaction with AP-2. More recent kinetic studies using MS indicated that EGFR phosphorylation at both Tyr998 (6) and Ser991 (10) occurs relatively slowly compared with other EGF-induced tyrosine phosphorylations known to be involved in receptor-proximal signal transduction. For example, Mann and co-workers (10) recorded maximal phosphorylation at EGFR sites Tyr1110, Tyr1172, and Tyr1197 at 1 min post-EGF, whereas EGF-stimulated phosphorylation at Tyr998 was still increasing at 15 min post-EGF (5), and a peptide containing both Ser(P)991 and Thr(P)993 peaked after 10 min (10). However, the role of phosphorylation at Tyr998 and Ser991 has not been reported. Another region of the EGFR, spanning residues 1026–1046, was identified by Zwang and Yarden (17) as a target of phosphorylation downstream of the stress-activated mitogen-activated protein (MAP) kinase p38 and associated with transient internalization and recycling of the EGFR in response to cytokine (TNFα) and stress challenges such as UV irradiation and the chemotherapeutic agent cisplatinum. Within this part of the receptor, a 13-residue section spanning 1029–1041 and the leucines at 1034 and 1035 in particular were found to be essential for ligand- and dimerization-induced EGFR endocytosis (18). Although both EGF- and stress-induced EGFR internalization may be clathrin-mediated, they differ in that the former leads to receptor down-regulation and involves the E3 ubiquitin ligase CBL (19), whereas the latter involves receptor recycling, is not associated with receptor phosphorylation at the CBL binding site Tyr(P)1069, and, in the case of TNFα treatment, involves activation of the transforming growth factor β-activated kinase TAK1 upstream of p38 (20). Interestingly although p38 kinase is not required for EGF-induced EGFR internalization, it is required for CBL-dependent receptor degradation (21). Therefore, alternate pathways involving p38 kinase regulate the down-regulation or recycling of the EGFR in response to diverse extracellular signals. However, the molecular details that govern these two processes are not fully understood.

In the current study, EGFR phosphorylation, signaling, protein-protein interactions, and trafficking were analyzed to address the role of Tyr998 and Ser991 in EGFR endocytosis. This was achieved by application of complementary methods including quantitative selected reaction monitoring (SRM, also known as MRM for multiple reaction monitoring) mass spectrometry, fluorescence imaging and cell sorting, immunoaffinity protein enrichment and blotting, and site-directed mutagenesis. Substitution mutations that prevented phosphorylation at EGFR Tyr998 and Ser991 did not prevent EGFR-to-ERK signaling but impaired EGF-induced receptor internalization and stimulated p38 kinase-dependent receptor phosphorylation at positions Ser1039 and Thr1041. These findings confirm the importance of Tyr998 and reveal a role for Ser991 in EGF-mediated EGFR internalization possibly involving crosstalk with the p38 kinase-dependent EGFR recycling pathway.

EXPERIMENTAL PROCEDURES

Constructs and Reagents—Site-directed mutagenesis was used to generate Tyr998-to-Phe and Ser991-to-Ala mutations in a construct encoding a chimeric EGFR-FLAG-GFP protein (22). For immunoprecipitation (IP) anti-FLAG-M2 was obtained from Sigma-Aldrich. For Western blotting, anti-FLAG and anti-Tyr(P) (4G10) were obtained from Sigma-Aldrich and Millipore (Billerica, MA), respectively. All other chemical reagents were purchased from Sigma-Aldrich; all other antibodies were obtained from Cell Signaling Technology (Danvers, MA). Toptips (200 μl) for preparing custom-made C18 tips were purchased from Canadian Life Science (Peterborough, Ontario, Canada). SB-202190 was from Sigma-Aldrich; all other chemical reagents were purchased from Sigma-Aldrich, and aqueous solutions were prepared using Milli-Q grade water (Millipore, Bedford, MA).

Cell Culture and Cell Lysis—Human embryonic kidney 293 (HEK293) cells and lines derived from them were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine serum. HEK-EGFR was derived from HEK293 and stably expresses chimeric EGFR as described previously (22). HEK293 derivatives stably expressing chimeric EGFR Y998F and S991A variants were

2 The convention for EGFR numbering includes the 24-residue signal sequence.
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EGFR expressing transfec-
tants were cultured as described above and including G418 (400 μg/ml). For IP and Western blotting, cells were deprived of serum overnight, treated with or without EGFR (100 ng/ml, 37 °C), then rinsed with ice-cold PBS, and lysed in Nonidet P-40 buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1% (v/v) Nonidet P-40, 100 mM NaF, 1 mM sodium orthovanadate, and protease inhibitors). In advance of MS analysis, prior to immunoprecipitation cell lysates were further clarified by ultracentrifugation for 60 min at 100,000 × g. For inhibition of p38 kinase, SB-202190 was used at a final concentration of 50 μM, a concentration at which it is not expected to affect ERK1/2, c-Jun NH2-terminal kinase (JNK), and several other related kinases (23).

Fluorescence Microscopy and Flow Cytometry—For fluorescence imaging cells were cultured to subconfluence, deprived of serum overnight, and then treated with or without EGF (100 ng/ml) at 4 °C and then at 37 °C for 0.5, 5, or 15 min. Cells were then fixed (3.7% paraformaldehyde, 23 °C, 10 min), washed with PBS, and observed by fluorescence microscopy as described previously (24).

For bioin labeling EGFR on the cell surface, cells were grown in 60-mm dishes to subconfluence, deprived of serum overnight, and then treated with or without EGF (50 ng/ml, 37 °C, 30 min). Biotinylation of intact cells was carried out as described by Zhang et al. (25). Briefly cells were suspended to 2.5 × 107 cells/ml in PBS (pH 8.0) and then incubated with 1.0 mg/ml sulfo-NHS-biotin (Pierce) at 23 °C for 30 min. Cells were then washed three times with PBS, lysed, and subjected to IP with immobilized anti-FLAG. Western blotting was performed as described below. The biotin detection agent was streptavidin-horseradish peroxidase.

For flow cytometry, cells at 80% confluence were deprived of serum overnight and then incubated with rhodamine-EGF at 4 °C for 30 min. After washing, cells were incubated at 37 °C for various time points (see Fig. 4B). After an acidic wash (0.2 M acetic acid and 0.5 M NaCl, pH 2.8) to remove non-internalized EGF, the fluorescence emission of internalized EGF was detected by flow cytometry (26).

IP and Western Blotting—For IP-Western analysis of associated proteins, 2 μg of total cellular protein in clarified lysates were extracted with immobilized anti-FLAG beads (10 μl) overnight at 4 °C. Immunoprecipitates were washed three times with Nonidet P-40 buffer, and proteins were eluted with 30 μl of 2× Laemmli sample buffer. Following SDS-PAGE proteins were electrophoretically transferred to Immobilon-P membranes (Millipore), then developed by Enhanced Chemiluminescence reagents, imaged with x-ray film (Eastman Kodak Co., XRP), and quantified by using a Fluor-S MultiImager (Bio-Rad). For direct Western blotting of lysates, 4 μg protein were loaded per lane.

For MS analysis of IP samples, 5-fold more starting material (by protein amount) and 30 μl of anti-FLAG beads were used. Immunoprecipitates were washed three times with Nonidet P-40 buffer and twice with HPLC water and then eluted with 50 μl of 0.15% TFA. Proteins were further processed as described previously (22) and then analyzed as described below.

Mass Spectrometry—Samples were analyzed on a linear ion trap Orbitrap hybrid analyzer (LTQ-Orbitrap, ThermoFisher, San Jose, CA) outfitted with a nanospray source and EASY-nLC split-free nano-LC system (Proxeon Biosystems, Odense, Denmark). The instrument method consisted of one MS full scan (400–1400 m/z) in the Orbitrap mass analyzer, an automatic gain control target of 500,000 with a maximum ion injection of 500 ms, one microscan, and a resolution of 60,000. Three data-dependent MS/MS scans were performed in the linear ion trap using the three most intense ions at 35% normalized collision energy. The MS and MS/MS scans were obtained in parallel fashion. In MS/MS mode automatic gain control targets were 10,000 with a maximum ion injection time of 100 ms. A minimum ion intensity of 1000 was required to trigger an MS/MS spectrum. The dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count with a repeat duration of 30 s and exclusion duration of 45 s.

SRM analysis was carried out by using a TSQ Quantum Ultra triple quadrupole MS instrument (Thermo Scientific) with a Proxeon source and LC system as described above except that the gradient time associated with the Orbitrap was 120 min and was 40 min with the TSQ instrument. Peptides were captured in a homemade 4-cm × 150-μm inner diameter trapping column and eluted over a 20-cm × 75-μm inner diameter fused silica analytical column. The packing material for both columns was Magic C18 (Michrom BioResources, Auburn, CA). The gradient was 0–65% acetonitrile in 0.1% formic acid over 35 min at 250 nl/min. The validated transitions for all phosphopeptides and unphosphorylated peptides for EGFR immunoprecipitates are listed in supplemental Table 1 and were monitored for the duration of the run. Most peptides were followed by using two unique transitions. Q2 gas pressure was 1.5 millitons, and collision energy was calculated relative to the precursor ion mass by the following formula: m/z × 0.034 V + 3 V. The scan width was 0.02 Da, and the dwell time was 10 ms. Q1 was operated at a resolution of 0.2, and Q3 resolution was 0.7. Xcalibur data acquisition software and SRM Builder software (Thermo Scientific) were used for data acquisition and total ion current calculations.

MS Data Analysis—Tandem mass spectra were extracted by BioWorks version 3.3. All MS/MS samples were analyzed using SEQUEST (ThermoFinnigan, San Jose, CA; version 27, revision 12) and X! Tandem (The Global Proteome Machine Organization; version 2006.04.01.2). Both search engines were set up to search the IPI human database (version 3.18, 60,090 entries) assuming trypsin digestion, allowing two missed cleavages, and using a fragment ion mass tolerance of 0.5 Da and a parent ion tolerance of 0.02 Da. The iodoacetamide derivative of cysteine was specified as a fixed modification. The phosphorylation of Tyr was specified in SEQUEST and the phosphorylation of Tyr, Ser, and Thr was specified in X! Tandem as variable modifications.

Scaffold (Proteome Software Inc., Portland, OR; version Scaffold-01.06.05) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (27). Protein identifications were accepted if they could be established at greater than 95.0% probability as assigned by the Protein Prophet algorithm (28). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The assignment of post-translational modifications was made by the search engines (described above) and verified manually. Modification by sulfation was not distinguishable from phosphorylation with the mass accuracy of these experiments. However, these isobaric modifications were assumed to be phosphorylation if anti-Tyr(P) enrichment was used for peptide purification.

Label-free relative quantification was approached by two methods. In the first, differential abundance was estimated based on spectral counts; i.e. the total number of peptides identified by MS/MS and assigned to a protein. For a given protein, the total non-phosphorylated peptides from four experiments were summed, and values were normalized according to non-phosphorylated EGFR peptide spectral counts (29, 30). In the second, peak areas of SRM transitions were determined by using SRM Builder prototype software (Thermo Scientific; version 1.0) after confirming for each peptide the co-elution of all transitions. The most abundant transition for each peptide was used for quantification unless interference in this channel was observed (32). For those cases, the second most abundant transition was used. Noise levels were estimated based upon visual inspection.

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Tyr998 is documented to be relatively slow compared with sis—
The accumulation of phosphorylation at EGFR residue of RAS-ERK signaling, was monitored by Western immunoblotting (38). As expected, phosphorylation at this position was rapid and reached an apparent maximum by 1 min (Fig. 1D). It was observed that even 90 min after EGF stimulation there was not yet appreciable down-regulation of EGFR (Fig. 1E), which is typically more apparent by 4 h post-EGF (21). The relatively slow accumulation of phosphorylation at Ser991 following EGFR treatment was pronounced and differs from the kinetics of the doubly phosphorylated isomer (Ser(P)991 with Thr(P)993) described by Olsen et al. (10) that reached a maximum abundance near 10 min post-EGF and then receded to basal levels by 20 min. However, it is possible that the kinetics of these phosphorylations differ as a function of cell type and receptor densities, which are parameters that are different in our study and the comprehensive study by Olsen et al. (10).

The SRM measurements of the Ser(P)991 peptide were normalized to the amount of a non-phosphorylated EGFR peptide YSFGATCVK (residues 285–293) from the same sample. The signal for this control peptide was generated by summation of transitions precursor → y6 and precursor → y7 at corresponding time points of EGF stimulation (supplemental Fig. 1). The EGFR control peptide produced a high signal (intensity >10⁶ units) and low coefficient of variation (CV = 8.3 ± 3.3%, n = 9) in test samples. Measurements of this peptide, therefore, were used as an indicator of EGFR amount in all SRM experiments in this study.

The relatively slow kinetics of phosphorylation at Ser991 and Tyr998 suggested that these modifications might not be involved in rapid signaling events associated with EGFR activation. To test this, site-directed mutagenesis was used to remove the side chain hydroxyl groups at these positions to prevent phosphorylation. Transfected HEK293 cell lines stably expressing variant proteins containing Tyr998-to-Phe (EGFR Y998F) and Ser991-to-Ala (EGFR S991A) at levels comparable to wild type (WT) EGFR in HEK-EGFR were established. These cell cultures were monitored for EGF-induced phosphorylation at Tyr1092 and for activation (phosphorylation) of the downstream MAP kinase ERK1/2. Phosphorylation of EGFR at Tyr1092 was unaffected by either Y998F or S991A substitution (Fig. 2, A and B), and the two mutant receptors did not show statistically significant changes in phosphorylated ERK1/2 following EGF treatment when compared with WT EGFR (Fig. 2, C and D).

To examine endocytosis of WT EGFR and the phosphorylation-defective receptor variants, localization of chimeric receptors containing carboxyl-terminal green fluorescent protein (GFP) was monitored by fluorescence microscopy. In serum-starved HEK-EGFR, HEK-S991A, and HEK-Y998F cells, the receptors were concentrated near the cell periphery consistent with plasma membrane localization (Fig. 3, left panels, long arrows). After exposure to 100 ng/ml EGF and incubation at 37 °C for 5 and 15 min, WT EGFR staining at the cell margins was diminished and became internalized and concentrated into intracellular aggregates characteristic of endosomes (Fig. 3, upper row, short arrows). By contrast,
FIG. 1. Detection and quantification of phosphorylation at EGFR Ser991 by SRM. A, trypsin-digested immunopurified EGFR was analyzed by high resolution LC-MS/MS (Proxeon LC/source; Thermo LTQ-Orbitrap). The MS/MS spectrum of the EGFR peptide MHLPpS991PTDSNFYR (where pS is phosphoserine) is shown (upper panel). The y and b series ions are indicated. Arrows point to product ions corresponding to parent − 98 and y10 − 98 ions (as indicated), which reflect the neutral loss of phosphoric acid from the Ser(P) side chain. B, SRM LC elution profiles plotting the summed signal intensities of ions corresponding to singly phosphorylated MHLPpSPTDSNFYR (precursor, m/z = 822.85, z = 2) transitioning to 1) precursor − 98/2 (m/z = 773.86, z = 2) and 2) y10 − 98 (m/z = 1165.50, z = 1) at various time points after EGF stimulation as indicated. C, histogram depicting the integrated SRM signal intensities normalized to a non-phosphorylated EGFR peptide in the same sample. Error bars indicate S.D. from three biological repeats of the experiment. D and E, Western blot (WB) analysis of whole-cell protein extracts from HEK-EGFR cells harvested following EGF treatment for the indicated durations. Antibodies specific to the EGFR phosphopeptide containing Tyr(P)1092 (D) or that recognize the FLAG epitope on EGFR-FLAG protein (E) were used to probe the filters, which were further developed with appropriate secondary antibody-enzyme conjugates and chemiluminescence imaging (see “Experimental Procedures”). pY, phosphotyrosine.
Y998F and S991A mutant receptors displayed far less inter-

nalization/aggregation and remained largely concentrated at

the cell periphery (Fig. 3, middle and lower rows, long arrows).

These observations demonstrate defective endocytosis of the

mutant receptors. The endocytosis defect of EGFR Y998F is

expected based on Sorkin et al. (16). The cell staining was
categorized into two types: WT-like, typified by internalized
and aggregated receptors and little or no staining at the cell

margins, and mutant-like, which lacked intracellular aggre-
gates and retained concentrated staining at the cell margins.

After tabulating these patterns in 100 cells in three separate
experiments, 60% of WT receptors displayed the WT-like
pattern of internalized receptors (diminished peripheral stain-
ing accompanied by internalized aggregates), whereas only
30% of Y998F and 25% of S991A receptors showed this

pattern.

To further examine and to quantify the extent of EGF-
stimulated receptor internalization, cells were treated with or
without EGF for 30 min, and then receptors exposed on the
cell surface were covalently labeled with biotin. Receptors
were then purified by immunoprecipitation and quantified for

biotinylation by Western blotting with a streptavidin probe

(Fig. 4A). After EGF treatment, the amount of surface-ex-
posed, biotinylated WT EGFR was decreased by ~50%,
whereas the amounts of surface exposed Y998F and S991A
receptors were almost equivalent to levels in untreated cells
(Fig. 4A). As an additional measure of receptor endocytosis,
receptor-mediated internalization of rhodamine-labeled EGF
was measured by flow cytometry. As shown in Fig. 4B, ligand
internalization by Y998F and S991A was less than that ob-
erved for WT EGFR. Therefore, by three different measures,
the Y998F and S991A variants were defective for receptor
endocytosis.

EGFR-associated Proteins and Phosphorylation—To further
address the function of the kinase domain proximal phospho-
rylation sites at Ser991 and Tyr998, receptor-associated pro-
teins and receptor phosphorylation were examined by using
LC-MS/MS and in some instances quantified by SRM. Tan-
dem MS analysis served three purposes. First, it enabled the
identification of receptor-associated proteins, which could then
be compared with a reference set of EGFR-associated proteins.
Second, it facilitated the measurement of spectral counts as a
first approximation of protein abundance (39). Third, it provided information on peptides and their fragmentations, which were useful in the design of SRM methods for the quantification of receptor-associated proteins in repeated experiments.

EGFR immunoprecipitates from EGF-stimulated cells expressing WT and variant EGFR proteins were prepared by using anti-FLAG antibodies and analyzed by LC-MS/MS for associated proteins similar to previously published methods involving gel-free, LC-MS/MS analysis of trypsin-digested, anti-FLAG immunoprecipitates (22, 40). In aggregate 444 proteins were identified in the anti-FLAG immunoprecipitates of WT and mutant receptors by using criteria described under “Experimental Procedures” (i.e. at least two distinct peptides and 95% probability scores by Peptide Prophet and Protein Prophet algorithms (27, 28)). Not surprisingly, this list (supplemental Table 1) contains proteins commonly known to associate with FLAG immunoprecipitates (40). To focus the analysis of receptor-associated proteins, the list was compared with data from the manually curated Human Protein Reference Database, which lists 144 EGFR-binding proteins that have experimental validation in the literature. Table I shows the 17 proteins, in addition to the EGFR, that intersect these two data sets including the ligand EGF; proteins involved in EGFR signaling such as SHC1, GRB2, and SOS1; and others associated with receptor endocytosis, including the ubiquitin E3 ligases CBL and CBLB and ubiquitin (Table I).

The spectral counts of these EGFR-associated proteins were noted as a crude measure of their abundance in the IP samples. Spectral counts were normalized by adjusting for the number of spectral counts from non-phosphorylated EGFR peptides. EGFR peptides, as expected, were by far the most abundant class of peptide in the samples (Table I), and this observation served to validate that the IP method was specific for this antigen. For each of the proteins mentioned above, VAV2, and the phosphatidylinositol 3-kinase p85 subunits (PIK3R1 and PIK3R2), spectral counts associated with WT EGFR increased as a consequence of EGF treatment of cells. Furthermore with the exception of EGF and CBLB, the spectral counts for these proteins measured with EGF-stimulated Y998F and S991A receptors were less than those observed for WT (Table I).

To quantify the receptor-associated proteins, including changes due to EGF stimulation and as a consequence of the
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Proteins identified in EGFR immunoprecipitates are listed according to their IPI number, protein name, and molecular weight as indicated. Percent sequence coverage and protein identification probability according to the Protein Prophet algorithm (29) are also shown. LC-MS/MS analysis (LTQ-Orbitrap) was performed with trypsin-digested EGFR immunoprecipitates from unstimulated WT EGFR-expressing cells (Cont) and EGF-stimulated cells expressing WT EGFR (EGFR), Y998F variant (Y998F), and S991A variant (S991A). Spectral counts (columns 6–9) are the total number of spectra, including repeat identifications, of peptides derived from the indicated proteins. The spectral counts in each column were normalized by a factor such that the number of unphosphorylated EGFR-derived peptides (shown in the top row) were equalized to the value obtained for the EGFR sample. The actual number of non-phosphorylated, receptor-derived peptides is shown in parentheses. Note that the accession number for EGF corresponds to the 1207-residue pre/pro-EGF protein, whereas the degree of coverage, correspondingly, the value obtained for the EGFR sample. The actual number of non-phosphorylated, receptor-derived peptides is shown in parentheses.

Y998F and S991A mutations, SRM analysis was applied to the receptor-immunoprecipitate complexes isolated from cells before and after EGF treatment. An SRM method that tracked the top six EGFR-associated proteins (according to spectral counts associated with WT EGFR; Table I) was formulated based on transitions summarized in supplemental Table 2. The fragmentation patterns obtained during discovery analyses with the LTQ-Orbitrap were a useful starting point toward the development of the SRM transitions. However, the resonance excitation-based fragmentations produced in the linear ion trap were not identical with the true CIDs undertaken in Q2 of the triple quadrupole TSQ instrument that was used for SRM. The intensity of b ions was generally greater in the linear ion trap, which also had a low molecular weight cutoff (i.e., 1⁄3 rule) that made low m/z ions more predominant in the triple quadrupole TSQ instrument. In addition, the LC gradients were of different durations (120 min with the Orbitrap and 40 min with the TSQ instrument). The SRM method was applied in three independent biological experiments resulting in the quantification of the EGFR and the six receptor-associated proteins (Fig. 5 and Table II). The EGFR peptide YSFAGATCVK (residues 285–293; described above), which is common to the WT and variant proteins and not subject to phosphorylation, was quantified and used to normalize for the amount of EGFR in each sample.

The interactions of all six proteins were verified by the SRM analysis (Fig. 5). The largest EGF-stimulated -fold increases in receptor binding were observed for CBL followed by GRB2. CBL association with WT EGF increased more than 7-fold following EGF treatment but was less than 5-fold with S991A and was ~4-fold with the Y998F variant. The drop in CBL association associated with the Y998F mutation was significantly different from WT (p = 0.01; Fig. 5). EGF-stimulated receptor association of GRB2 was roughly 3-fold, which was a statistically significant increase over that observed without EGF treatment, and the measurements for GRB2 were not different between WT and the variant receptors (Fig. 5). The associations of VAV2 and SOS1 were measured to be essentially constitutive and unaffected by the phosphorylation site mutations because no statistically significant differences were noted following EGF treatments or between the WT and the variant receptors (Fig. 5). The binding of SHC1 to WT EGFR was stimulated more than 2-fold by EGF, but for the receptor variants (Fig. 5). The interactions of all six proteins were verified by the SRM analysis (Fig. 5). The largest EGF-stimulated -fold increases in receptor binding were observed for CBL followed by GRB2. CBL association with WT EGF increased more than 7-fold following EGF treatment but was less than 5-fold with S991A and was ~4-fold with the Y998F variant. The drop in CBL association associated with the Y998F mutation was significantly different from WT (p = 0.01; Fig. 5). EGF-stimulated receptor association of GRB2 was roughly 3-fold, which was a statistically significant increase over that observed without EGF treatment, and the measurements for GRB2 were not different between WT and the variant receptors (Fig. 5). The associations of VAV2 and SOS1 were measured to be essentially constitutive and unaffected by the phosphorylation site mutations because no statistically significant differences were noted following EGF treatments or between the WT and the variant receptors (Fig. 5). The binding of SHC1 to WT EGFR was stimulated more than 2-fold by EGF, but for the receptor variants (Fig. 5). The binding of SHC1 to WT EGFR was stimulated more than 2-fold by EGF, but for the receptor variants (Fig. 5).
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compared with WT (Fig. 5). Table II shows the peptides, SRM transitions, and coefficients of variance associated with the data depicted in Fig. 5. A more complete listing of these measurements including SRM intensities for each of the three repeated experiments is provided in supplemental Table 4.

The observed changes in receptor-associated CBL and ubiquitin were supported by Western blot analysis (Fig. 6). The variant receptors were not generally impaired for phosphorylation at the CBL binding site Ser(P)\textsuperscript{1069}, but the Y998F and S991A protein complexes captured by anti-FLAG IP were observed to contain less CBL and ubiquitin than did WT (Fig. 6). The co-immunoprecipitation of ubiquitin may reflect the ubiquitination of any of the proteins in the receptor (anti-FLAG) immunoprecipitates. However, prominent anti-ubiquitin immunostaining was concentrated at or slightly above the migration of the receptors, suggesting that ubiquitination of the receptors, as expected (19), may account for a portion of the ubiquitin measured by MS.

The level of endogenous EGFR in HEK293 cells is very minor (22), and association with other ErbB family receptors was not detected. This indicated that dimerization of FLAG-tagged ectopic receptors with endogenous ErbB family members to which associated proteins might bind was not a factor in these experiments.

Among the peptides characterized by LC-MS/MS analysis of EGFR immunoprecipitates were phosphopeptides. Following EGFR stimulation, the total number of spectral counts for EGFR-derived phosphorylated peptides was 69 for WT EGFR, 72 for the Y998F variant, and 73 for S991A. Thus, by this estimation the mutants missing one or another kinase domain proximal phosphorylation site were not generally impaired for phosphorylation. As shown in Table III, there is evidence for phosphorylation at 18 sites on the receptor, including three Thr(P), six Tyr(P), and nine Ser(P). Additional information related to these assignments including protein search algorithm scores and MS/MS spectra are included in supplemental Table 3 and supplemental Fig. 2. The low number of observed phosphopeptides was not surprising because no phosphopeptide enrichment was used in this analysis. For several sites, the number of observed spectra was too low to warrant further discussion.

Surprisingly in the region spanning EGFR residues 1000–1052 there was evidence for increased phosphorylation with the Y998F and S991A variants (see bold region in Table III). There were, respectively, 20 and 23 phosphorylated peptides identified by spectral counting for these variants but only six

TABLE II

| Protein | Peptide | Q1/Q3 transition | WT Mean | CV | S.E. | Y998F Mean | CV | S.E. | S991A Mean | CV | S.E. |
|---------|---------|------------------|--------|----|-----|------------|----|-----|------------|----|-----|
| CBL     | GTEPIVDPFDPR | 721.37/631.3 | 7.46   | 0.46 | 1.97 | 4.21       | 0.55 | 1.33 | 4.86       | 0.42 | 1.19 |
| GRB2    | FNSLNEIVDVRH | 753.87/802.4 | 3.15   | 0.23 | 0.42 | 3.10       | 0.45 | 0.81 | 2.99       | 0.33 | 0.57 |
| SHC1    | GILLVPALK   | 405.78/428.3  | 2.85   | 0.31 | 0.51 | 1.87       | 0.42 | 0.45 | 2.21       | 0.59 | 0.75 |
| VAV2    | HLLVDPGEQVR | 673.89/656.4  | 1.40   | 0.19 | 0.16 | 1.51       | 0.19 | 0.17 | 1.38       | 0.39 | 0.31 |
| UBB     | SHAGSYTULIR | 552.79/880.5  | 3.28   | 0.33 | 0.63 | 3.80       | 0.26 | 0.57 | 2.49       | 0.36 | 0.52 |
| SOS1    | TITLEVEPSDVIENVK | 894.47/1131.6 | 1.91 | 0.28 | 0.31 | 1.63       | 0.25 | 0.23 | 1.53       | 0.41 | 0.36 |
EGFR Phosphorylation Sites Regulate Receptor Endocytosis

The monophosphorylated isobaric isomers containing either Ser(P)1039 or Thr(P)1041 were analyzed by LC-MS/MS, which confirmed their identities. For example, their y12 fragment ions were clearly distinctive (Fig. 7, A and B). The two species did not co-elute during reverse phase chromatography, and this facilitated their resolution and ion current measurement by high resolution LC-MS (Fig. 7C). To facilitate the quantification of these species by SRM, the measurement of transitions involving distinctive y ions was attempted but proved inefficient. Therefore, an optimized SRM method was developed that included neutral loss of phosphoric acid (i.e. doubly charged parent − 98/2) and y5-associated transitions, which were common to the two phosphoisomers and efficiently detected (Fig. 7, A, B, and D). Note that different elution gradients were used for the LC-MS and SRM analyses shown in Fig. 7, C and D, respectively, so the retention times were not identical. The Orbitrap MS extracted ion current chromatograph and triple quadrupole SRM traces were similar (Fig. 7, compare C and D), and several repeats of the LC-MS/MS experiment indicated that the Ser(P)1039 peptide consistently eluted immediately before the Thr(P)1041 isomer.

Having established the SRM method, it was applied to quantify phosphorylation at Ser1039 and Thr1041 and address three questions: first, to determine whether these modifications were affected by EGF ligand; second, to determine whether they were elevated with the Y998F and S991A variant receptors compared with WT as implied by the semiquantitative spectral counting analysis shown in Table III; and third, to determine whether these modifications require p38 kinase. Stress (e.g. TNFα or UV irradiation)-induced p38 kinase-dependent phosphorylation within the Ser/Thr-rich segment of the EGFR spanning residues 1026–1046, but not at Ser1039 specifically, was shown by Zwang and Yarden (17). Fig. 7, E and F, present a summary of three independent repeats of an experiment that addressed these questions. Stimulation of cells with EGF caused an ~3-fold elevation in the abundance of the Ser(P)1039 peptide (3.4 ± 1.3 S.D.) (Fig. 7E), whereas both the Y998F and S991A variants showed a roughly 10-fold elevation in the abundance of the Ser(P)1039 peptide after EGF treatment. The pronounced EGF-stimulated increase in Ser(P)1039 was largely eliminated by treatment of cells with SB-202190, which is a potent inhibitor of MAP kinases p38α/β (23), indicating that these phosphorylation sites are regulated by p38 kinase (Fig. 7E). A similar phenomenon was observed for Thr(P)1041 wherein EGF treatment stimulated Thr(P)1041 more than 2-fold in WT (2.7 ± 1.1 S.D.) but to an even greater extent with the variants (Fig. 7F). The Thr1041 modifications were also sensitive to the p38 inhibitor (Fig. 7F).

**DISCUSSION**

**EGFR Mutants Y998F and S991A Signal to ERK but Are Defective in Endocytosis**—Cellular responses to EGFR activation occur with different kinetics, and to some extent this reflects temporal regulation of receptor and substrate phosphorylation and the dynamics of receptor trafficking and protein-protein interactions (3, 9, 10). The phosphorylations of EGFR at Ser991 and Tyr998 were found in tumors as sites modulated by EGF and an EGFR kinase inhibitor, suggesting their involvement in EGFR regulation and/or signaling (10, 12). In this study and other published reports (10) EGF-stimulated phosphorylations at these sites occurred with relatively slow kinetics and in this study were found not to be required for downstream ERK activation, which suggests that their function is not required for signaling along the RAS-ERK axis.
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Indeed the results from three different assays indicated that the phosphorylation-deficient variants S991A and Y998F were defective in receptor endocytosis. These data indicate that some extent signaling may proceed independently of normal receptor trafficking.

Our data indicate that EGFR endocytosis is subject to regulation involving the phosphorylation of Tyr998 and amino-terminal to it at Ser991. Sorkin et al. (16) described Tyr998 as part of a tyrosine-based internalization motif. Indeed the structural analysis by Owen and Evans (41) of an EGFR peptide containing this tyrosine-based internalization motif. Indeed the structural analysis by Owen and Evans (41) of an EGFR peptide containing this tyrosine-based internalization motif (FYRALM) bound to the endocytosis adaptor protein AP-2 as a requisite for clathrin-mediated endocytosis (42). We did not identify adaptins as EGFR-interactions with adaptin AP-2 as a requisite for clathrin-mediated endocytosis defect associated with the variant receptors nor the extent to which this phenotype is a consequence of the side chain substitutions or their lack of phosphorylation but the phosphorylations at Tyr998 and Ser991. Given evidence that EGFR endocytosis does not require EGFR kinase activity (26), we predict that the Tyr998 kinase is not the EGFR itself. Again this is consistent with Tyr998 phosphorylation accumulating after the initial wave of EGFR autophosphorylation events (10).

Quantification of EGFR-associated Proteins and Phosphorylation by SRM—The results obtained by SRM-based examination of a limited set of receptor-associated proteins are consistent with the conclusion that the S991A and Y998F variants are defective in EGFR endocytosis. Among the six well established EGFR-associated proteins examined, the ubiquitin E3 ligase CBL and ubiquitin itself were found to differ from WT with one or the other endocytosis-defective receptor variant. Ubiquitin and CBL have defined roles in the down-regulation of EGFR and other receptor tyrosine kinases (19, 43–45). Western blot analysis confirmed the diminished association of CBL with the activated variant receptors com-
FIG. 7. Detection and quantification of phosphorylation at EGFR Ser1039 and Thr1041 by LC-MS/MS and SRM. A and B, trypsin-digested immuno (anti-FLAG) purified EGFR proteins were analyzed by high resolution LC-MS/MS. MS/MS spectra of the EGFR peptide TPLLSSSASp1039TSNNTACIDR (where pS is phosphoserine) (A) and TPLLSSLSAp1041TSNNTACIDR (where pT is phosphothreonine) (B) are shown. The y and b series ions are indicated. Arrows point to product y12 ions. As expected the m/z values of y12 ions are different between Ser(P)1039 (A) and Thr(P)1041 (B) peptides because Thr1041 or Thr(P)1041 is the amino-terminal residue in the y12 ions. C, the orbitrap XIC for the isobaric phosphopeptides with m/z 1144.04 and containing Ser(P)1039 or Thr(P)1041, as indicated, are shown to be chromatographically resolved. D, SRM LC elution profiles plotting the summed signal intensities of ions corresponding to singly phosphorylated TPLLSSLSATSNNSTACIDR (precursor, m/z = 1144.04, z = 2) transitioning to 1) precursor − 98/2 (m/z = 1095.05, z = 2) and 2) y5 (m/z = 634.30, z = 1) as indicated. E and F, histograms depicting the integrated SRM signal intensities of Ser(P)1039 peptide (E) and Thr(P)1041 peptide (F) normalized to a control EGFR peptide in the same sample (see “Experimental Procedures”). EGFR, Y998F, and S991A cell cultures were treated without (Control) or with (+EGF) EGF (100 ng/ml, 15 min, 37 °C) or with EGF after a 30-min pretreatment with the p38 inhibitor SB-202190 (10 μM) (+EGF + SB). Error bars indicate S.D. from three biological repeats of the experiment. For statistical analysis, Y998F and S991A samples were compared with wild type EGFR in EGF-stimulated samples (+EGF), and EGFR, Y998F and S991A samples in SB-202190 treated cells (+EGF + SB) were compared with themselves in EGF-stimulated samples (+EGF). *, p = 0.05 compared with wild type EGFR + EGF. In all cases phosphopeptides were statistically significantly less (p < 0.05) for control and SB-202190-treated samples compared with cognate + EGF samples. XIC, extracted ion current.
pared with WT and demonstrated that the mutant receptors remained competent for phosphorylation at Tyr$^{1069}$, which is the direct binding site on the receptor for CBL. GRB2 is required for EGFR endocytosis (24, 46–49). In addition, it interacts with SHC and SOS1/2 as effectors of RAS and ERK downstream of activated tyrosine kinases (50). The EGFR variants Y998F and S991A retained both EGF-stimulated phosphorylation at the principal GRB2 binding site Tyr$^{1092}$ (38) and GRB2 association in amounts not significantly different from WT. This further indicates that the early stages of ligand-stimulated receptor activation were not impaired by the Y998F and S991A mutations and that the endocytosis defects associated with Y998F and S991A are not a consequence of impaired GRB2 bindings to the receptors.

The EGF-stimulated increase in receptor-associated ubiquitin displayed by the variant receptors (although for S991A it was less than that of WT) is consistent with the conclusion that to some extent they did become internalized. However, our data do not address which proteins in the receptor complexes were covalently modified by ubiquitin.

A Link between EGFR Recycling and Degradation Pathways—Our data revealed an unforeseen connection between the pathways of EGFR recycling and down-regulation. The p38 MAP kinase-dependent phosphorylations at Ser$^{1039}$ and Thr$^{1041}$ in the endocytosis-defective variants were pronounced. Because the phosphorylations at Ser$^{1039}$ and Thr$^{1041}$ were EGF-stimulated, we conclude they are a normal part of the EGF response and that the Y998F and S991A mutations do not themselves constitute a “stress” that induced the recycling response in cells. Unmapped p38-dependent phosphorylation(s) in a 21-residue segment of the receptor (spanning 1026–1046) that includes these residues was shown previously to be required for EGFR recycling following cell treatments including TNF$\alpha$, osmotic stress, UV irradiation, and the chemotherapeutic agent cisplatinum but was not linked to EGF ligand-stimulated EGFR endocytosis (17). Indeed p38 kinase is not required for EGF-stimulated EGFR internalization but is required subsequently for CBL-mediated ubiquitination and degradation of internalized EGFR (21).

We speculate that the phosphorylations at positions Ser$^{991}$ and Tyr$^{998}$ that accompany EGFR activation by ligand are normally involved in dampening the levels of phosphorylation at Ser$^{1039}$ and Thr$^{1041}$ as part of a mechanism to prevent receptor recycling and promote receptor destruction. In this model, the endocytosis defects associated with the S991A and Y998F variants may be due in part to inappropriate phosphorylation at Ser$^{1039}$ and consequently uncoordinated trafficking of internalized receptors.

In conclusion, our results identify the Ser$^{991}$ and Tyr$^{998}$ phosphorylation sites as important determinants in ligand-stimulated EGFR endocytosis linked to p38 MAP kinase-dependent receptor phosphorylation at Ser$^{1039}$ and Thr$^{1041}$. A better understanding of the mechanism by which these receptor phosphorylations are regulated and coordinated may reveal further insight into the molecular mechanisms that govern receptor recycling and degradation, which are important determinants in EGFR-directed cancer therapeutics (3, 51).

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**REFERENCES**

1. Schlessinger, J. (2002) Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. Cell 110, 669–672

2. Zhang, X., Gureasko, J., Shen, K., Cole, P. A., and Kuriyan, J. (2006) An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell 125, 1137–1149

3. Yarden, Y. (2001) The EGFR family and its ligands in human cancer. Signalling mechanisms and therapeutic opportunities. Eur. J. Cancer 37, Suppl. 4, S3–S8

4. Jones, R. B., Gordus, A., Kralj, J. A., and MacBeath, G. (2006) A quantitative protein interaction network for the ErbB receptors using protein microarrays. Nature 439, 168–174

5. Schulze, W. X., Deng, L., and Mann, M. (2005) Phosphotyrosine interaction of the ErbB receptor kinase family. Mol. Syst. Biol. 1, 2005.0008

6. Blagoev, B., Ong, S. E., Kratchmarova, I., and Mann, M. (2004) Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. Nat. Biotechnol. 22, 1139–1145

7. Zhang, Y., Wolf-Yadlin, A., Ross, P. L., Pappin, D. J., Rush, J., Lauffenburger, D. A., and White, F. M. (2005) Time-resolved mass spectrometry of tyrosine phosphorylation sites in the epidermal growth factor receptor signaling network reveals dynamic modules. Mol. Cell. Proteomics 4, 1240–1250

8. Wolf-Yadlin, A., Hautaniemi, S., Lauffenburger, D. A., and White, F. M. (2007) Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. Proc. Natl. Acad. Sci. U.S.A. 104, 5860–5865

9. Dengjel, J., Akimov, V., Olsen, J. V., Bunckenborg, J., Mann, M., Blagoev, B., and Andersen, J. S. (2007) Quantitative proteomic assessment of very early cellular signaling events. Nat. Biotechnol. 25, 566–568

10. Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648

11. Wu, S. L., Kim, J., Bandle, R. W., Liotta, L., Petricoin, E., and Karger, B. L. (2006) Dynamic profiling of the post-translational modifications and interaction partners of epidermal growth factor receptor signaling after stimulation by epidermal growth factor using Extended Range Proteomic Analysis (ERPA). Mol. Cell. Proteomics 5, 1610–1627

12. Stover, D., Caldwell, J., Marto, J., Root, K., Mestan, J., Stumm, M., Ornatsky, O., Orsi, C., Radosevic, N., Liao, L., Fabbro, D., and Moran, M. F. (2004) Differential phosphoprofiles of EGF and EGFR kinase inhibitor-treated human tumor cells and mouse xenografts. Clin. Proteomics 1, 69–80

13. Heisermann, G. J., and Gill, G. N. (1988) Epidermal growth factor receptor threonine and serine residues phosphorylated in vivo. J. Biol. Chem. 253, 13152–13158

14. Boeri Erba, E., Matthiesen, R., Bunkenborg, J., Schulze, W. X., Di Stefano, P., Cabodi, S., Tarone, G., Delfippi, P., and Jensen, O. N. (2007) Quantitation of multisite EGF receptor phosphorylation using mass spectrometry and a novel normalization approach. J. Proteome Res. 6, 2768–2785

15. Thelemann, A., Petti, F., Griffin, G., Iwata, K., Hunt, T., Settinari, T., Fenyo, D., Gibson, N., and Haley, J. D. (2005) Phosphotyrosine signaling net-
works in epidermal growth factor receptor overexpressing squamous carcinoma cells. Mol. Cell. Proteomics 4, 356–376
16. Sorkin, A., Mazzotti, M., Sorkina, T., Scotto, L., and Beguinot, L. (1996) Epidermal growth factor receptor interaction with clathrin adaptors is mediated by the Tyr974-containing internalization motif. J. Biol. Chem. 271, 13377–13384
17. Zhang, Y., and Yarden, Y. (2006) p38 MAP kinase mediates stress-induced internalization of EGFR: implications for cancer chemotherapy. EMBO J. 25, 4195–4206
18. Wang, Q., Zhu, F., and Wang, Z. (2007) Identification of EGFR receptor C-terminal sequences 1005–1017 and di-leucine motif 1010LL1011 as essential in EGFR receptor endocytosis. Exp. Cell Res. 313, 3349–3363
19. Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S., and Sorkin, A. (2006) Differential regulation of EGFR receptor internalization and degradation by multiquitubination within the kinase domain. Mol. Cell 21, 737–748
20. Singhirunnusorn, P., Ueno, Y., Matsuo, M., Suzuki, S., Sakai, I., and Sakurai, H. (2007) Transient suppression of ligand-mediated activation of epidermal growth factor receptor by tumor necrosis factor-alpha through the TAK1-p38 signaling pathway. J. Biol. Chem. 282, 12698–12706
21. Frey, M. R., Dise, R. S., Edeblum, K. L., and Polik, D. B. (2008) p38 kinase regulates epidermal growth factor receptor downregulation and cellular migration. EMBO J. 25, 5683–5692
22. Tong, J., Taylor, P., Vojceva, E., St-Germain, J. R., Jin, L. L., Nikolic, A., Gu, X., Li, Z. H., Trudel, S., and Moran, M. F. (2008) Tandem immunoprecipitation of phosphotyrosine-mass spectrometry (TIPy-MS) indicates C19orf119 becomes tyrosine phosphorylated and associated with activated epidermal growth factor receptor. J. Proteome Res. 7, 1067–1077
23. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. 351, 95–105
24. Wang, Z., and Moran, M. F. (1996) Requirement for the adapter protein Grb2 and Shc on the epidermal growth factor receptor. Mol. Biol. Cell 7, 18928–18933
25. Wang, Q., Zhu, F., and Wang, Z. (2007) Identification of EGFR phosphorylation sites Regulate Receptor Endocytosis. J. C. (2005) Multiple reaction monitoring as a method for identifying protein phosphorylation: the phosphate-dependent phosphorylation profile (2006) A quantitative results-driven approach to analyzing multisite protein phosphorylation. J. Proteome Res. 5, 10296–10301
26. Ewing, R. M., Chu, P., Elisma, F., Li, H., Taylor, P., Clemie, S., McEwain-Cerajewski, L., Robinson, M. D., O’Connor, L., Li, M., Taylor, R., Dharsee, M., Ho, Y., Heilbut, A., Moore, Z., Zhang, S., Ornatsky, O., Buxhan, Y., Cheng, Y., Shier, M., Sheng, Y., Milescu, J., Abu-Farha, M., Lambert, J. P., Duevel, H. S., Stewart, I. M., Kuehl, B., Hogue, K., Colwill, K., Gladwish, K., Muskat, B., Kinach, R., Adams, S. L., Moran, M. F., Morin, G. B., Topaloglou, T., and Figeys, D. (2007) Large-scale mapping of human protein-protein interactions by mass spectrometry. Mol. Syst. Biol. 3, 89
27. Ewing, R. M., Chu, P., Elisma, F., Li, H., Taylor, P., Clemie, S., McEwain-Cerajewski, L., Robinson, M. D., O’Connor, L., Li, M., Taylor, R., Dharsee, M., Ho, Y., Heilbut, A., Moore, Z., Zhang, S., Ornatsky, O., Buxhan, Y., Cheng, Y., Shier, M., Sheng, Y., Milescu, J., Abu-Farha, M., Lambert, J. P., Duevel, H. S., Stewart, I. M., Kuehl, B., Hogue, K., Colwill, K., Gladwish, K., Muskat, B., Kinach, R., Adams, S. L., Moran, M. F., Morin, G. B., Topaloglou, T., and Figeys, D. (2007) Large-scale mapping of human protein-protein interactions by mass spectrometry. Mol. Syst. Biol. 3, 89
28. Wang, Q., Zhu, F., and Wang, Z. (2007) Identification of EGFR phosphorylation sites Regulate Receptor Endocytosis. J. C. (2005) Multiple reaction monitoring as a method for identifying protein phosphorylation: the phosphate-dependent phosphorylation profile (2006) A quantitative results-driven approach to analyzing multisite protein phosphorylation. J. Proteome Res. 5, 10296–10301
29. Grandal, M. V., and Madshus, I. H. (2008) Epidermal growth factor receptor interaction with clathrin adaptors is required to ubiquitylate and degrade EGFR. EMBO J. 27, 1327–1336
30. Topaloglou, T., and Figeys, D. (2007) Large-scale mapping of human protein-protein interactions by mass spectrometry. Mol. Syst. Biol. 3, 89
31. Owen, D. J., and Evans, P. R. (1998) A structural explanation for the recognition of tyrosine-based endocytotic signals. Science 282, 1327–1332
32. Sorkin, A., Kuhn, E., Carr, S. A., and Boyland, H. (2003) Cbl-mediated ubiquitination is required for lysosomal sorting of epidermal growth factor receptor but is dispensable for endocytosis. J. Biol. Chem. 278, 28950–28960
33. Duan, L., Miura, Y., Dimri, M., Majumder, B., Dode, I. L., Reddi, A. L., Ghosh, A., Fernandes, N., Zhou, P., Mullane-Robinson, K., Rao, N., Dongphue, S., Rogers, I., Soprano, K., Band, V., and Band, H. (2003) Cbl-mediated ubiquitinylation is required for lysosomal sorting of epidermal growth factor receptor but is dispensable for endocytosis. J. Biol. Chem. 278, 28950–28960
34. Huang, F., and Sorkin, A. (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
35. Umezawa, K., Tsukada, M., and Yoshimori, T. (2008) UbC45 and c-Cbl continue to ubiquitinate EGF receptor after internalization to facilitate polyubiquitination and degradation. Mol. Biol. Cell 19, 3454–3462
36. Waterman, H., Katz, M., Robin, C., Shlateglum, K., Lavi, S., Elson, A., Jovin, T., and Yarden, Y. (2002) A mutant EGF-receptor defective in ubiquitylation and endocytosis unrolls a role for Grb2 in negative signaling. EMBO J. 21, 303–313
37. Martinu, L., Santiago-Walker, A., Qi, H., and Chou, M. M. (2002) Endocytosis of epidermal growth factor receptor regulated by Grb2-mediated recruitment of the Rab5 GTPase-activating protein RH1. J. Biol. Chem. 277, 50996–51002
38. Yamazaki, T., Zaal, K., Hailey, D., Presley, J., Lippincott-Schwartz, J., and Samelson, L. E. (2002) Role of Grb2 in EGF-stimulated EGFR internalization. J. Cell Sci. 115, 1791–1802
39. Jiang, X., Huang, F., Marusyk, A., and Sorkin, A. (2003) Grb2 regulates internalization of EGF receptors through clathrin-coated pits. Exp. Cell Res. 289, 2858–2869