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UBPY-mediated Epidermal Growth Factor Receptor (EGFR) De-ubiquitination Promotes EGFR Degradation*[S]

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Whereas poly-ubiquitination targets protein substrates for proteasomal degradation, mono-ubiquitination is known to regulate protein trafficking in the endosomal system and to target cargo proteins for lysosomal degradation. The role of the de-ubiquitinating enzymes AMSH and UBPY in endosomal trafficking of cargo proteins such as the epidermal growth factor receptor (EGFR) has only very recently been the subject of study and is already a matter of debate. Although one report (Mizuno, E., Iura, T., Mukai, A., Yoshimori, T., Kitamura, N., and Komada, M. (2005) *Mol. Biol. Cell* 16, 5163–5174) concludes that UBPY negatively regulates EGFR degradation by de-ubiquitinating the EGFR on endosomes, another report (Row, P. E., Prior, I. A., McCullough, J., Clague, M. J., and Urbe, S. (2006) *J. Biol. Chem.* 281, 12618–12624) concludes that UBPY-mediated EGFR de-ubiquitination is essential for EGFR degradation. Here, we demonstrate that Usp8/UBPY, the mammalian ortholog of budding yeast Ubp4/Doa4, constitutively co-precipitates in a bivalent manner with the EGFR. Moreover, UBPY is a substrate for Src-family tyrosine kinases that are activated after ligand-induced EGFR activation. Using overexpression of three different recombinant dominant negative UBPY mutants (UBPY C748A mutant, UBPY 1–505, and UBPY 640–1080) in NIH3T3 and HEK293 cells, we demonstrate that UBPY affects both constitutive and ligand-induced (i) EGFR ubiquitination, (ii) EGFR expression levels, and (iii) the appearance of intermediate EGFR degradation products as well as (iv) downstream mitogen-activated protein kinase signal transduction. Our findings provide further evidence in favor of the model that UBPY-mediated EGFR de-ubiquitination promotes EGFR degradation.

The EGFR[2] (or ErbB1) is a member of the ErbB growth factor receptor-tyrosine kinase family that is involved in the genesis of many human solid tumors (for review, see Refs. 1 and 2). EGFR activation leads to multiple downstream signaling events that control various cellular processes such as mitogenesis, cell survival, differentiation, and cell migration. These processes must be tightly regulated to prevent uncontrolled mitogenic signaling, cell survival, and cell migration, which may lead to tumor formation. The principal mechanism by which cells down-regulate activated receptors is by ligand-mediated endocytosis and subsequent delivery of the activated ligand-receptor complex to the lysosome for degradation (3, 4).

Targeting of cell-surface receptors to the lumen of the lysosome is a complex process that is tightly regulated by several protein complexes along distinct steps of the endocytic pathway (3, 5). After ligand-induced dimerization of the EGFR, phosphorylated tyrosine residues in the C-terminal tail serve as docking sites for SH2 or PTB (phosphotyrosine binding) domain-containing proteins. The Cbl family of E3 ubiquitin ligases (6) is recruited to EGFR Tyr(P)-1045 through its variant SH2 domain (7). The activated EGFR is then internalized through clathrin-coated pits and transported to early endosomes after which cargo is sorted either to the recycling pathway or to the lysosome for degradation (for review, see Refs. 8). Lysosomal targeting involves the delivery of cargo proteins to internal vesicles of late endosomes/multivesicular bodies (MVBs), which subsequently fuse with pre-existing lysosomes for subsequent degradation (5). Formation of MVBs is dependent on the evolutionary conserved vacuolar protein sorting (Vps) class E proteins including Hrs/Vps27, ESCRT-I, -II, and -III complexes, Vps31/Bro1/Alix, and the AAA-ATPase Vps4/SKD1 (5).

Several lines of evidence strongly support involvement of ubiquitin in the endosomal trafficking of cargo proteins, including sorting of cargo proteins at the trans-Golgi network, internalization of cargo proteins at the plasma-membrane, sorting of cargo proteins to the MVB pathway, and budding of retroviral particles at the plasma-membrane (9). Indeed, many of the key players (*e.g.* Eps15, GGAs, Hrs, Vps23/TSG101, Vps36, Vps9 proteins) in the endocytic pathway contain ubiquitin-interacting domains that assist them in recognizing ubiquitinated cargo and/or endosomal adapter proteins (*e.g.* Eps15, CIN85) (9).

After agonist stimulation various activated protein-tyrosine kinases, including receptor-tyrosine kinases and Src-family

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.

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[2] The abbreviations used are: EGFR, epidermal growth factor (EGF)receptor; Vps, vacuolar protein sorting; MVB, multivesicular bodies; SH, Src homology; SB, SH3 binding; dSB, deletion mutant lacking the SB motif(s); NEM, *N*-ethylmaleimide; Usp, ubiquitin-specific protease; Hbp, Hrs-binding protein; IP, immunoprecipitation; IB, immunoblotting; Rhod, Rhodanese domain; WCL, whole cell lysates; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; wt, wild type; HEK cells, human embryonic kidney cells; E3, ubiquitin ligase.

kinases, are rapidly ubiquitinated by Cbl E3 ubiquitin ligases (6). Recruitment of Cbl to EGFR Tyr(P)-1045 leads to EGFR mono-ubiquitination on multiple lysines (referred to as multi-ubiquitination) (10, 11) as opposed to poly-ubiquitination, which targets cargo proteins for proteasomal degradation. Recent data obtained with mass spectrometry suggest, however, that a significant fraction of the EGFR is modified with K63-linked poly-ubiquitin chains within the kinase domain (12). The role of EGFR ubiquitination during the internalization phase is debated (see for instance Ref. 13). This controversy may be due to redundancy of ubiquitin-dependent and -independent (*e.g.* clathrin AP2-mediated) internalization signals (14), multivalent recruitment of Cbl to the EGFR resulting in low level ubiquitination of the EGFR Y1045F mutant (15), and perhaps other factors. Accumulating evidence suggests, however, a role of EGFR ubiquitination in lysosomal targeting (13). Here, binding of Hrs to ubiquitinated cargo proteins through its ubiquitin interacting motif (16) and recruitment of the ESCRT-I complex to the surface of endosomes (17) initiates the lysosomal targeting of EGFR proteins. Ubiquitinated cargo is then transferred to the ESCRT-I protein TSG101/Vps23, which interacts with ubiquitin through its catalytically inactive ubiquitin conjugating enzyme variant (UEV) domain (18). Binding of the ESCRT-I complex to the surface of endosomes leads to the sequential recruitment of ESCRT-II, Vps31/Alix, and ESCRT-III complexes. In budding yeast, this supramolecular assemblage facilitates the recruitment of the de-ubiquitinating enzyme Ubp4/Doa4 (19) in an ESCRT-III- and Vps31/Bro1-dependent manner (20–22). Doa4 action allows the recycling of ubiquitin from cargo proteins (23) before the translocation of cargo proteins into internal vesicles of MVBs (20, 24). The evolutionary conserved Vps4/SKD1 AAA-ATPase activity is required to release the endosomal protein complex from the membrane, thereby triggering MVB formation and incorporation of cargo into internal vesicles of MVBs (5).

We have previously shown that ubiquitinated EGFRs undergo de-ubiquitination before their delivery to lysosomes (4). Although our studies were considered elsewhere, several groups have recently implicated the JAMM/MPN+ family member AMSH (associated molecule with the SH3 domain of Stan) and the ubiquitin-specific protease (Usp) family member Usp8/UBPY in regulation of EGFR ubiquitination and degradation, with conflicting conclusions. Although one report (25) concludes that UBPY negatively regulates EGFR degradation by de-ubiquitinating the EGFR on endosomes, another report (26) concludes that UBPY-mediated EGFR de-ubiquitination is essential for EGFR degradation. Here, using overexpression of three different recombinant dominant negative UBPY mutants in NIH3T3 and HEK293 cells, we investigate the role of UBPY in EGFR ubiquitination and downstream signaling.

EXPERIMENTAL PROCEDURES

Reagents—The following monoclonal (mAb) and polyclonal (pAb) antibodies were used in this study: anti-EGFR 528 mAb (used for immunoprecipitation (IP)) and anti-EGFR 1005 pAb (used for immunoblotting (IB) except when indicated) (Santa Cruz), anti-EGFR Ab-12 pAb (Neomarkers), anti-phosphotyrosine 4G10 mAb (Upstate Biotechnology), anti-ubiquitin pAb, and anti-FLAG M2 mAb (Sigma), anti-active (phospho-p44/p42) MAPK mAb (Cell Signaling), anti-GFP antibody (Santa Cruz), anti-GAPDH antibody (Abcam), as well as goat anti-rabbit (GARPO) and goat anti-mouse (GAMPO) mAbs linked to horseradish peroxidase (Signal Transduction Laboratories). Other reagents that were used in this study included protein A and protein G-Sepharose beads (Amersham Biosciences), PP2 Src kinase inhibitor, PD153035 EGFR-tyrosine kinase inhibitor (Calbiochem), Lipofectamine 2000 (Invitrogen), and mouse EGF (BD Bioscience). Anti-UBPY polyclonal antibodies (W39) were kindly provided by Dr. E. Martegani (27). The following expression vectors were used: pEGFP vector (Clonetech), pZome-1N retroviral vector (Cellzome), pLZRS-IRES-GFP (kindly provided by Dr. J. Jansen Department of Hematology, UMCN, Radboud University Nijmegen), pcDNA3-EGFR wild type and Y1045F (kindly provided by Dr. Y. Yarden), and pME18s-FLAG UBPY wild-type and dSB constructs (kindly provided by Dr. N. Kitamura) (28). The Phoenix ecotrophic packaging cell line was obtained from Dr. G. P. Nolan (Stanford University Medical Center) through the American Type Culture Collection (ATCC).

DNA Manipulation and Plasmid Construction—pMe18s-FLAG-UBPY (corresponding to wild-type (wt) full-length mouse UBPY amino acids 1–1080) and pMe18s-FLAG-UBPY-dSB (UBPY lacking both N-terminal and C-terminal Hrs-binding protein (Hbp) SH3 binding (SB) motifs) were kindly provided by Dr. N. Kitamura (28). These constructs were used as the template to generate catalytically inactive FLAG-UBPY C748A and FLAG-UBPY-dSB-C748A. Wild-type and mutant UBPY cDNAs were then cloned into EcoR1 and SmaI sites of pEGFP-c2 vector to generate EGFP-UBPY wt, EGFP-UBPY-dSB, EGFP-UBPY-CA, and EGFP-UBPY-dSB-CA (Fig. 1). N-terminal and C-terminal deletion mutants were generated by amplifying individual domains of UBPY and subsequent cloning into EcoRI and SmaI sites of pEGFP-c2, which resulted in the following constructs: EGFP-Rhod (amino acids 1–504), EGFP-Rhod-dSB (amino acids 1–504 lacking the N-terminal SB motif), EGFP-Rhod-D140 (amino acids 140–504), EGFP-Rhod-dSB-D140 (amino acids 140–504 lacking the N-terminal SB motif), EGFP-Dub (amino acids 640–1080), EGFP-Dub-dSB (amino acids 640–1080 lacking the C-terminal SB motif), EGFP-Dub-CA (amino acids 640–1080 carrying the C748A mutation), and EGFP-Dub-dSB-CA (double mutant carrying both C-terminal dSB motif deletion and C748A mutation) (Fig. 1). The wild-type and C748A mutant of the Dub domain were also cloned into EcoRI and HindII sites of pZome-1N retroviral vector to generate protein A-tagged Dub domains. Finally, pMe18s-FLAG-UBPY constructs were digested with XhoI and SmaI to release UBPY sequence carrying the FLAG tag sequence and subsequently cloned into XhoI and SnaBI sites of pLZRS-IRES-GFP-PURO retroviral vector to create pLZRS-FLAG-UBPY-IRES-GFP constructs. All constructs and mutations were verified by nucleotide sequencing. Additional cloning strategies and primer sequences are available upon request.

Cell Culture and Generation of Stable Cell Lines—HER-14 (NIH3T3 cells stably transfected with the human EGFR), human embryonic kidney cells (HEK293), and Phoenix cell lines were grown at 37 °C in Dulbecco's Modified Eagle's

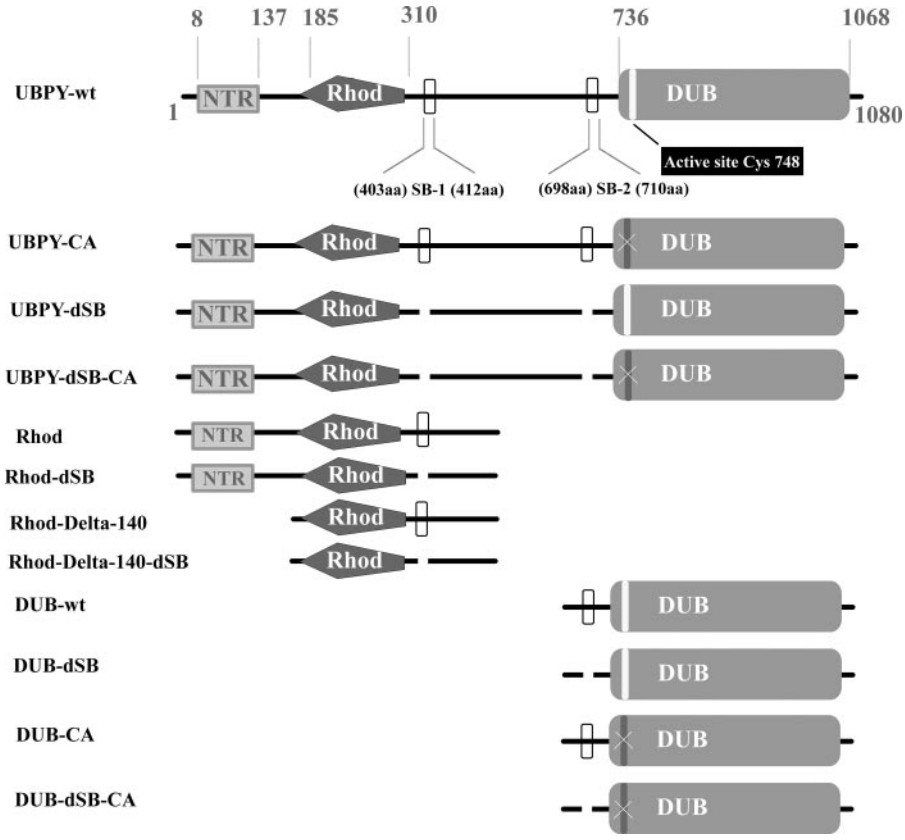

FIGURE 1. **Domain structure UBPY and mutants used in this study.** Amino acid boundaries of individual domains are indicated on *top* of the *figure*. *NTR*, N-terminal region; *DUB*, catalytic de-ubiquitination domain; *CA*, C748A catalytically inactive mutant; *Delta-140*, deletion mutant lacking amino acids 1–140; aa, amino acids.

medium supplemented with 10% newborn calf serum. Transient transfection was performed using either calcium phosphate precipitation or Lipofectamine 2000 according to standard/manufacturer's instructions. HER-14 cells stably expressing FLAG-UBPY (wt and dSB) or vector control were generated by Lipofectamine transfection with pLZRS-FLAG-UBPY-IRES-GFP constructs followed by puromycin selection. Stable HER-14 cell lines expressing protein A-tagged Dub domains or vector control were generated by transfection of Phoenix cells with pZome-1N-Dub-wt and -Dub-CA constructs, isolation of recombinant retrovirus, and viral infection of HER-14 cells followed by puromycin selection according to standard protocols.

Analysis of Proteins and Data Quantification—Cells were serum-starved 24 h post-transfection in Dulbecco's modified Eagle's medium containing 0.1% newborn calf serum plus 0.1% bovine serum albumin for 18 h before stimulation. Cells were stimulated with EGF (100 ng/ml) for 60 min unless specified otherwise. Cell lysis, immunoprecipitation, SDS-PAGE electrophoresis, and Western blotting was carried out as described previously (4). Signal quantification was performed with Photoshop software (Adobe systems). Briefly, mean pixel value was calculated for a predefined rectangle encompassing signal of interest after subtracting the background signal. Similar data calculation was carried out for loading control samples (*e.g.* GAPDH) as indicated in figure legends. The data plotted in the graphs represent mean pixel value (MPV) of signal of interest divided by the MPV of the loading control.

In Vitro De-ubiquitination Assay—FLAG-UBPY wt, and FLAG-UBPY C748A mutant were each transfected in two 10-cm plates of NIH3T3 by means of Lipofectamine. Cells were harvested 24 h post-transfection. FLAG-UBPY was immunoprecipitated with M2 anti-FLAG antibody coupled to Prot-G beads for 1.5 h at 4 °C. After extensive washing, beads were then split into two fractions. One was treated with *N*-ethylmaleimide (NEM) and the other with carrier ethanol at room temperature; their concentrations were kept constant throughout the experiment. Four 10-cm dishes of HER-14 cells were serum-starved overnight. To induce ubiquitination of EGFR, cells were stimulated with 100 ng/ml EGF for 15 min. Cells were then lysed on ice, and immunoprecipitation of ubiquitinated EGFR was performed with anti-EGFR (clone 528) coupled to protein A beads at 4 °C for 1.5 h. Beads were then washed extensively in lysis buffer and split into two fractions. EGFR beads were then reconstituted in Dub buffer (10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA). EGFR immunoprecipitates were then combined with UBPY immunoprecipitates in a 100-μl final volume of Dub buffer. The de-ubiquitination reaction was initiated by incubating the bead mixtures at 37 °C for 90 min with regular mixing. Beads were then washed 3 times with Tris-buffered saline with Tween and twice with phosphate-buffered saline. Then, Laemmli sample buffer was added to the mixture and incubated at 100 °C for 3 min before loading on SDS-PAGE and Western blotting.

RESULTS

Tyrosine Phosphorylation of UBPY after EGFR Activation—Based on the function of the yeast de-ubiquitinating enzyme Doa4/Ubp4, we hypothesized that Usp8/UBPY, the mammalian ortholog of Doa4, might be involved in de-ubiquitination of the EGFR. UBPY is the only mammalian Dub enzyme that, like Doa4, contains a catalytically inactive Rhodanese-like homology domain (29). In addition, phylogenetic analysis of fungal and metazoan ubiquitin-specific protease enzymes reveals that fungal Ubp4/Ubp5 paralogs form a single clade with metazoan Usp8 orthologs.[3] To assess the role of UBPY in EGFR signaling and turnover, we first asked whether UBPY is tyrosine-phosphorylated in response to EGF treatment. As seen in Fig. 2*A*, a clear EGF-induced tyrosine phosphorylation of FLAG-tagged UBPY was observed in NIH3T3 cells co-transfected with UBPY and EGFR, demonstrating that transfected UBPY is a substrate

[3] H. A. J. Alwan and J. E. M. van Leeuwen, unpublished results.

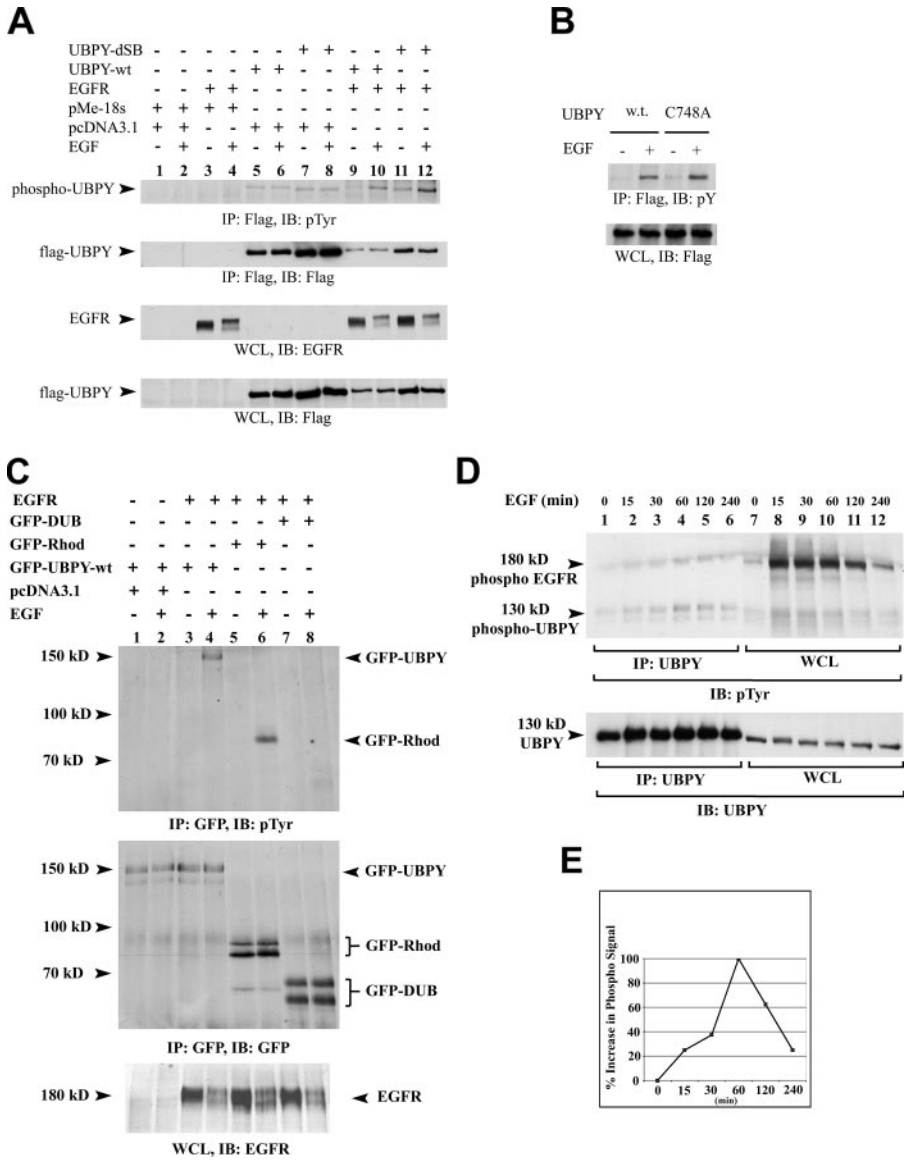

FIGURE 2. **Tyrosine phosphorylation of UBPY following EGFR activation.** *A*, NIH-3T3 cells were transfected with FLAG-tagged UBPY constructs, EGFR, or control vector (pME18s, pcDNA3. 1) as indicated. Serum-starved cells were EGF-stimulated for 1 h and lysed, and WCL were used for IP and IB with anti-FLAG, anti-phosphotyrosine (*pTyr*), and anti-EGFR antibodies as indicated. *B*, NIH3T3 cells were co-transfected with EGFR and either wild-type UBPY or catalytically inactive C748A mutant. Serum-starved cells were EGF-stimulated for 1 h, and WCL were used for IP and IB as indicated. *pY*, phosphotyrosine. *C*, HEK293 cells were transfected with EGFR, EGFP-tagged UBPY constructs, or vector control as indicated. Serum-starved cells were EGF-stimulated, and WCL were used for IP and IB as indicated. *D*, serum-starved HER-14 cells were EGF-stimulated for various periods of time as indicated. WCL were used for IP and IB with anti-UBPY and anti-phosphotyrosine as indicated. *E*, densitometric analysis of the phospho-signal of UBPY. Phospho-signal of the IPs in *D* was related to the amount of precipitated UBPY in the sequential blot and plotted as a function of time. Data are representative of 2–4 experiments.

for EGF-activated protein-tyrosine kinases. It should be noted that EGFR activation is associated with a characteristic mobility shift, as seen in Fig. 2*A* (*third panel*). UBPY is known to interact with Hbp through the Hbp SH3 binding motifs of UBPY (at amino acid positions 405–413 (SB-N) and at amino acids 700–708 (SB-C)) (28). However, as shown in Fig. 2*A*, EGF-induced tyrosine phosphorylation of FLAG-UBPY-dSB was similar compared with wild-type FLAG-UBPY, demonstrating that the Hbp SH3 binding motifs in UBPY are dispensable for UBPY tyrosine phosphorylation. UBPY contains a highly conserved active site cysteine (Cys-748) that is required for its enzymatic activity (30). As shown in Fig. 2*B*, UBPY C748A contained similar levels of phosphotyrosine compared with wild-type UBPY, demonstrating that UBPY de-ubiquitination activity is also not required for its tyrosine phosphorylation. To unequivocally demonstrate that the tyrosine-phosphorylated protein detected in UBPY precipitates is UBPY itself and not a co-migrating protein, we generated EGFP fusion constructs of wild-type, N-terminal-truncated, and C-terminal-truncated UBPY proteins. HEK293 cells were co-transfected with UBPY wt, UBPY 1–504 (which will be referred to as the Rhod (Rhodanese) construct), or UBPY 640–1080 (which will be referred to as the "Dub" construct). As shown in Fig. 2*C*, EGF stimulation induced an EGFR-dependent tyrosine phosphorylation of GFP-UBPY wt (155 kDa) and GFP-Rhod (85 kDa) but not of GFP-Dub even though it was efficiently expressed (*middle panel*) and its de-ubiquitinating enzyme activity was still intact (data not shown). These findings demonstrate that transfected UBPY itself undergoes tyrosine phosphorylation in response to EGFR activation and that the N-terminal Rhod but not the C-terminal Dub construct is sufficient for EGF-induced UBPY tyrosine phosphorylation. Moreover, as shown in Fig. 2*D*, we could also detect EGF-induced tyrosine phosphorylation of endogenous UBPY (130 kDa), which was precipitated from HER-14 cells and detected by Western blot using the anti-UBPY W39 polyclonal antiserum (27). Quantification of the UBPY phosphotyrosine signal showed that UBPY phosphorylation reached a maximum at 60 min after stimulation followed by a gradual decline until it reached near basal levels after 4 h of stimulation (Fig. 2*E*). Interestingly, a tyrosine-phosphorylated protein that co-migrates with the EGFR co-precipitated with endogenous UBPY (Fig. 2*D*, *lanes 1–6*), suggesting that UBPY associates with the EGFR. Collectively, these data demonstrate that (i) EGF induces UBPY tyrosine phosphorylation in an EGFR-dependent manner, (ii) UBPY is tyrosine-phosphorylated in its N-terminal half, and (iii) neither the Hbp SH3 binding motifs in the proline rich domain of UBPY nor the de-ubiquitination activity of UBPY is required for UBPY tyrosine phosphorylation.

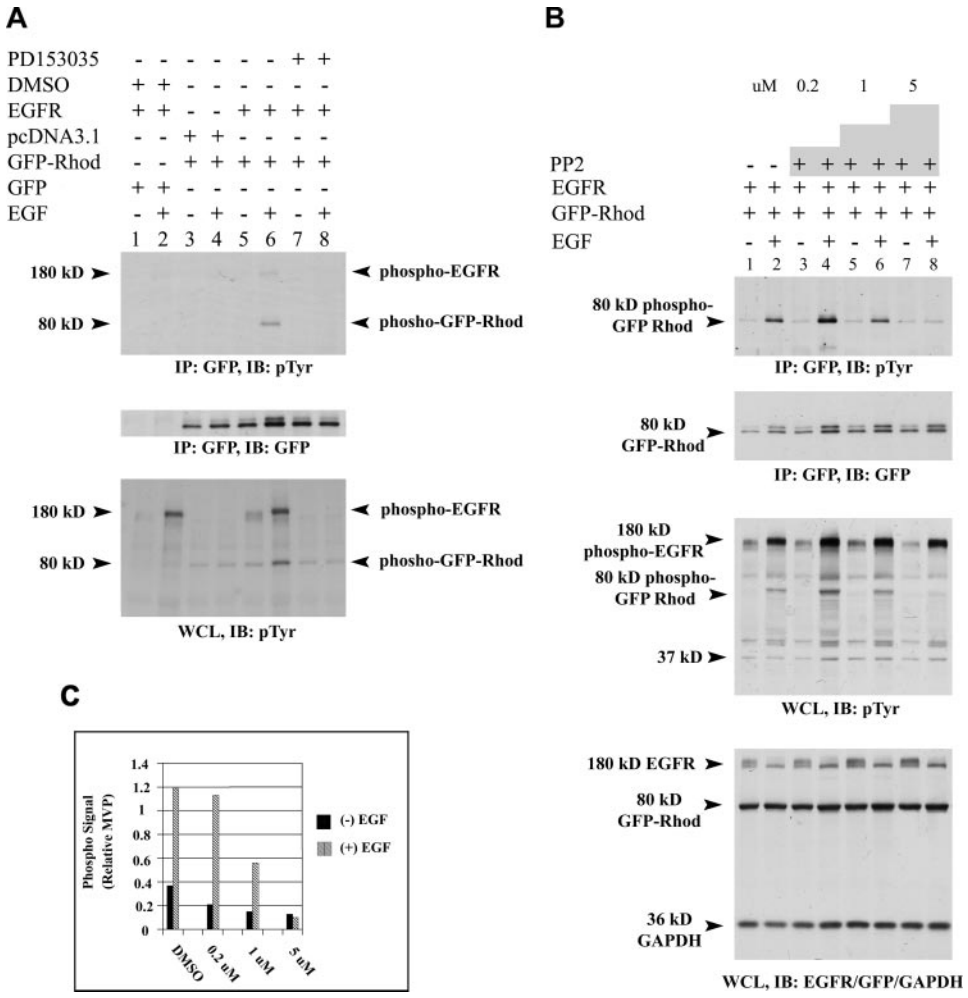

FIGURE 3. **Inhibition of Src kinases with PP2 blocks UBPY phosphorylation.** *A*, HEK293 cells were co-transfected with EGFR or pcDNA3.1 and GFP-Rhod or control pEGFP-c2 vector (*GFP*). Serum-starved cells were pretreated for 60 min with either 10 μM EGFR-tyrosine kinase inhibitor PD153035 or carrier Me_2SO (*DMSO*) and EGF-stimulated for 1 h. WCL were used for IP and IB as indicated. *pTyr*, phosphotyrosine. *B*, HEK293 cells were co-transfected with GFP-Rhod and EGFR. Serum-starved cells were pretreated for 60 min with increasing amounts of PP2 Src kinase inhibitor and EGF-stimulated for 1 h as indicated. WCL were used for IP and IB as indicated. *C*, densitometric analysis of phosphorylated GFP-Rhodanese signal in resting and EGF-stimulated cells as shown in *B*, *top panel*. Specific GFP-Rhod phospho-signal was related to the amount of precipitated GFP-Rhod as shown in *B*, (*2nd panel* from *top*) and plotted as a function of PP2 concentration. Dat are representative of 2–3 experiments.

Inhibition of Src Kinases with PP2 Blocks UBPY Tyrosine Phosphorylation—To further test whether the kinase activity of the EGFR is required for UBPY tyrosine phosphorylation, we used the specific EGFR kinase inhibitor PD153035. The EGFR kinase inhibitor effectively blocked EGF-induced tyrosine phosphorylation of the EGFR and other downstream signaling molecules as seen on WCL (Fig. 3*A*, *lower panel*). Importantly, EGF-induced tyrosine phosphorylation of the immunoprecipitated EGFP-Rhod construct was completely blocked in the presence of PD153035 (Fig. 3*A*, *top panel*). A tyrosine-phosphorylated band of around 180 kDa, most probably the EGFR, co-precipitated with EGFP-Rhod and disappeared (as expected) upon PD153035 treatment.

It is well known that Src-family tyrosine kinases are activated after EGFR activation (1, 31). To test whether Src-family tyrosine kinases may be responsible for UBPY tyrosine phosphorylation, we used the specific Src-family kinase inhibitor PP2. Significantly, EGF-induced GFP-Rhod tyrosine phosphorylation was reduced in a concentration-dependent manner by PP2 (Fig. 3, *B*, *top panel*, *C*). Complete inhibition of GFP-Rhod tyrosine phosphorylation was achieved with 5 μM PP2 (Fig. 3, *B* and C) even though tyrosine phosphorylation of the EGFR remained relatively unaffected (Fig. 3*B*, *third panel*). It should be noted that a slight decrease in EGFR tyrosine phosphorylation upon PP2 treatment is expected because the EGFR itself is a substrate for Src-family tyrosine kinases (31). These data demonstrate that UBPY is a substrate for Src-family tyrosine kinases and indicate that direct phosphorylation of UBPY by the EGFR kinase domain does not occur to a significant extent.

UBPY Is Found in a Complex with the EGFR—The data illustrated in Figs. 2 and 3 suggest that the tyrosine-phosphorylated EGFR co-precipitates with transfected as well as endogenous UBPY proteins. Indeed, as shown by direct co-precipitation (Fig. 4*A*), FLAG-UBPY co-precipitated with the EGFR. FLAG-UBPY-dSB bound the EGFR to similar levels as the wild-type UBPY (Fig. 4*A*), demonstrating that the Hbp SH3 binding motifs are not required for formation of a complex between UBPY and EGFR *in vivo*. Interestingly, FLAG-UBPY C748A showed a 4-fold enhanced binding to the EGFR compared with wild-type UBPY (Fig. 4*A*). The enhanced binding of the UBPY C748A mutant to the EGFR suggests that the stability of the UBPY-EGFR complex is enhanced when the catalytic activity of UBPY is abolished, indicating substrate trapping characteristics of the UBPY C748A mutant. Similar results were obtained with GFP-tagged mutants of UBPY (data not shown). To determine whether complex formation of UBPY with the EGFR is dependent on its kinase activity, we transfected FLAG-UBPY C748A (because of its enhanced binding to the EGFR) together with EGFR in the presence or absence of the EGFR kinase inhibitor PD153035. UBPY binding to the EGFR remained unaffected when cells cultured in low serum were treated with PD153035 (Fig. 4*B*, *top panel*) despite the fact that the kinase inhibitor dramatically decreased the activation state of the EGFR (Fig. 4*B*, *pTyr*). We conclude that UBPY constitutively forms a complex with the EGFR *in vivo* through a mechanism that is independent of the Hbp SH3 binding motifs and independent of EGFR kinase activity. Furthermore, the enhanced binding of the catalytically inactive C748A mutant of UBPY

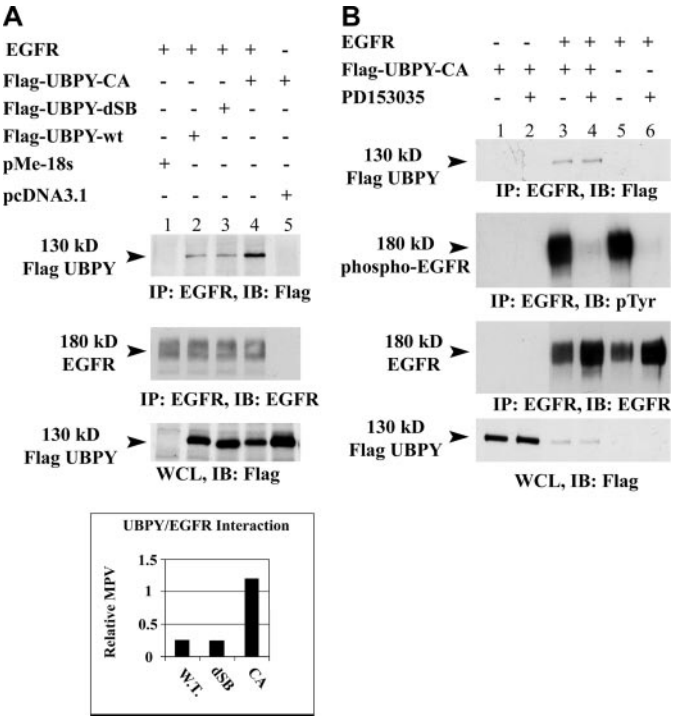

FIGURE 4. **UBPY constitutively associates with the EGFR in a kinase activity-independent manner.** *A*, HEK293 cells were co-transfected with EGFR, FLAG-tagged UBPY, or control vectors as indicated. Serum-starved (0.1% newborn calf serum) cells were lysed, and WCL were used for anti-EGFR IP and IB with anti-FLAG and anti-EGFR as indicated. The association between the EGFR and various UBPY constructs was quantified by means of densitometric analysis of FLAG signal in EGFR IPs (*upper panel*) relative to FLAG signal in WCL (*lower panel*). *B*, HEK293 cells were co-transfected with FLAG-UBPY C748A and EGFR or control pcDNA31. Serum-starved (0.1% newborn calf serum) cells were treated for 90 min with either 10 μM EGFR-tyrosine kinase inhibitor or carrier Me_2SO. WCL were used for anti-EGFR IP and IB with anti-FLAG, anti-phosphotyrosine, and anti-EGFR as indicated. Data are representative of 2–3 experiments.

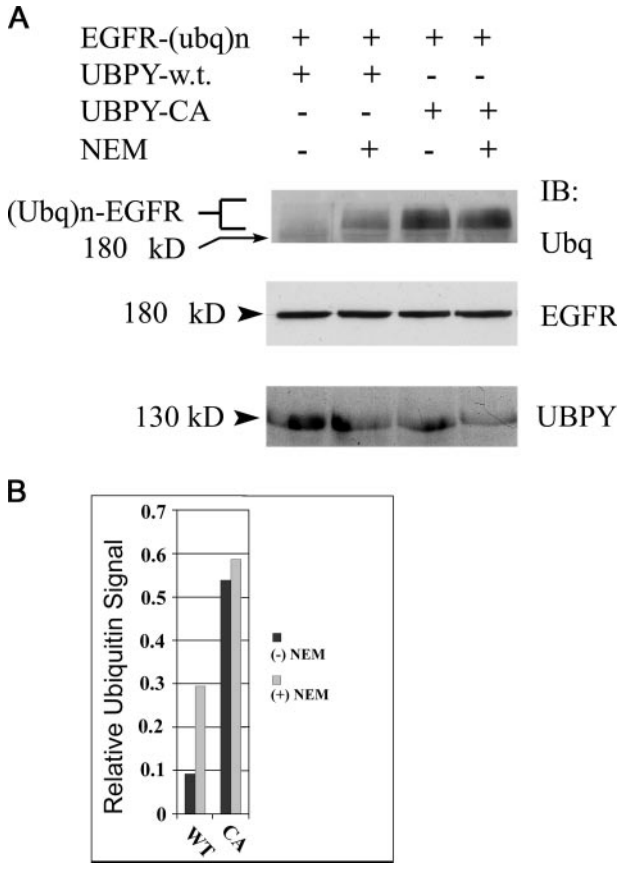

FIGURE 5. **UBPY de-ubiquitinates the EGFR *in vitro*.** *A*, for a detailed description of the procedure, see "Experimental Procedures." *B*, band intensities of the ubiquitin signal were calculated relative to the EGFR signal according to methods described under "Experimental Procedures." Data are representative of two experiments. *Ubq*, ubiquitin.

to the EGFR suggests that this mutant shows a substrate trap phenotype.

The EGFR Is a Substrate for UBPY De-ubiquitination Activity in Vitro—Having shown that UBPY is tyrosine-phosphorylated in response to EGFR activation and is constitutively associated with EGFR, we tested the impact of this interaction on the ubiquitination status of the EGFR. Initially, we tested whether the EGFR is a substrate for UBPY *in vitro*. Toward this goal, an *in vitro* EGFR de-ubiqutination assay was developed (see "Experimental Procedures"). Thus, FLAG-UBPY and FLAG-UBPY C748A were immuno-purified and tested for their ability to de-ubiquitinate immuno-purified ubiquitinated EGFRs. To measure the de-ubiquitination activity we used NEM, an alkylating agent that is highly potent in blocking de-ubiquitinating enzymes by covalent alkylation of their active site cysteine as a control. The difference in EGFR ubiquitination seen in NEM-treated *versus* untreated samples provides a measure for the de-ubiquitination activity during incubation of the samples at 37 °C. After the de-ubiquitination assay, samples were run on SDS-PAGE for a relatively short time to narrow the smear of ubiquitinated EGFRs to a relatively compact region, allowing better quantification. Untreated wild-type FLAG-UPBY was able to de-ubiquitinate the EGFR (Fig. 5, *A*, *first* and *second lanes*, and *B*). In contrast, FLAG-UBPY C748A failed to de-ubiquitinate the EGFR in the absence of NEM (Fig. 5, *A*, *third* and *fourth lanes*, and *B*).

The EGFR Is a Substrate for UBPY De-ubiquitination Activity in Vivo—To determine whether the EGFR is a substrate for UBPY de-ubiquitination activity *in vivo*, HER-14 cells stably expressing human EGFR were transfected with wild-type and catalytically inactive UBPY, transfectants were enriched by puromycin selection, and resistant cells were assayed for EGFR ubiquitination (Fig. 6*A*). Even though the expression of the C748A mutant was significantly less than wild-type UBPY (Fig. 6*A*, *bottom panel*), the UBPY C748A mutant markedly enhanced accumulation of ubiquitinated EGFRs after EGF stimulation (Fig. 6*A*, *top panel*). In our previous study we used IP-recapture procedures to demonstrate that the high molecular weight ubiquitinated species seen in EGFR IP represent ubiquitinated EGFRs (4). To further analyze the effect of UBPY C748A expression on EGFR steady state ubiquitination (*i.e.* in the absence of EGF), we co-transfected the EGFR with either UBPY wild-type or C748A mutant into HEK293 cells (Fig. 6*B*). Overexpression of wild-type UBPY did not markedly affect EGFR ubiquitination relative to vector control, but overexpression of the C748A mutant strongly enhanced accumulation of ubiquitinated EGFRs (Fig. 6*B*). Moreover, introduction of the dSB mutation in UBPY C748A mutant did not abolish the dominant interfering activity of the catalytically inactive UBPY (data

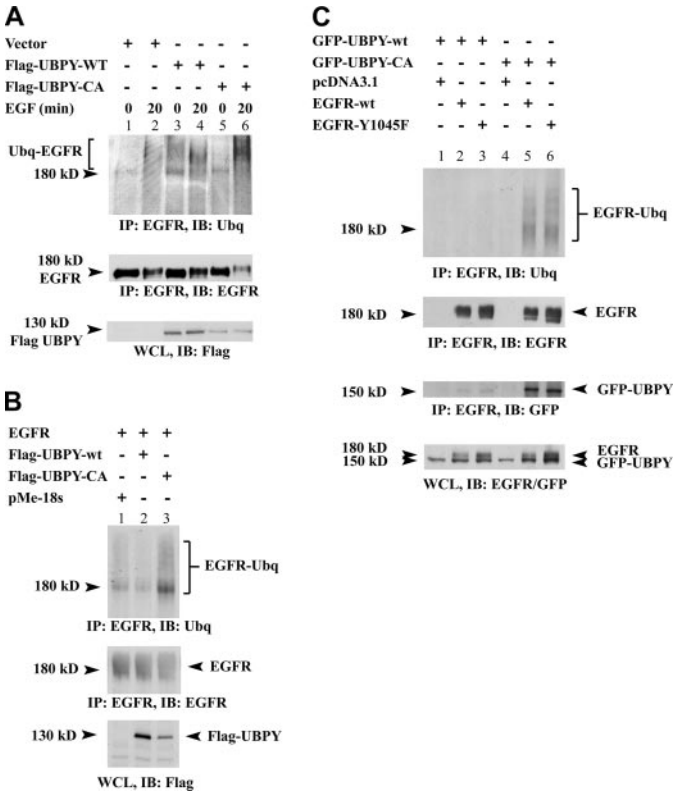

FIGURE 6. **UBPY de-ubiquitinates the EGFR *in vivo*.** *A*, HER-14 cells were transfected with FLAG-UBPY wt, C748A, or vector control (pLZRS-IRES-GFP) carrying the puromycin resistance gene and subjected to 5 days of puromycin selection. Serum-starved cells were stimulated with EGF for 20 min and lysed, and WCL were used for IP with anti-EGFR and IB with anti-ubiquitin, anti-EGFR (Ab12), and anti-FLAG as indicated. *Ubq*, ubiquitin. *B*, HEK293 cells were co-transfected with EGFR and FLAG-UBPY wt, C748A, or control vector as indicated. Serum-starved cells were lysed, and WCL were used for anti-EGFR IP and anti-ubiquitin, anti-EGFR (Ab12), and anti-FLAG IB as indicated. *C*, HEK293 cells were co-transfected with EGFR wt, Y1045F, or control vector and either GFP-UBPY wt or GFP-UBPY C748A as indicated. Serum-starved cells were lysed, and WCL were used for anti-EGFR IP and anti-ubiquitin, anti-EGFR (Ab12), and anti-GFP IB as indicated. Data are representative of two experiments.

not shown), indicating that the effect of C748A is not mediated through competition with AMSH for the Hbp SH3 domain. We conclude that overexpression of the full-length C748A catalytically inactive UBPY mutant leads to enhanced accumulation of ubiquitinated EGFRs in both ligand-independent as well as ligand-dependent model systems.

Ligand-induced EGFR multi-ubiquitination is critically dependent on the Cbl family of E3 ubiquitin ligase adapter proteins that is recruited to tyrosine-phosphorylated EGFR Tyr(P)-1045 (6). The ability of the enzymatically inactive UBPY C748A mutant to up-regulate EGFR ubiquitination under steady state conditions in HEK293 cells (*i.e.* in the absence of ligand) prompted us to investigate the effect of wild-type and C748A mutant UBPY on the ubiquitination of the EGFR Y1045F mutant, which shows a profound block in EGF-induced EGFR ubiquitination (Ref. 7 and data not shown). Most interestingly, the UBPY C748A mutant markedly enhanced steady state ubiquitination of both EGFR wt and EGFR Y1045F mutant constructs, whereas wild-type UBPY did not affect their ubiquitination status relative to vector control (Fig. 6*C*, *top panel*). These findings indicate that the steady state EGFR ubiquitination is determined by the balance between ubiquitination and de-ubiquitination. Consistent with our previous data, GFP-UBPY C748A mutant also showed enhanced binding to the EGFR Y1045F mutant when compared with GFP-UBPY wt. Collectively, the data in Fig. 6 demonstrate that UBPY modulates the EGFR ubiquitination status *in vivo* in both ligand-dependent and ligand-independent model systems. We further conclude that the recruitment of UBPY to the EGFR does not require phosphorylation of EGFR Tyr-1045, consistent with our experiments using EGFR kinase inhibitors (Fig. 4*B*). Moreover, these data demonstrate that the EGFR undergoes constitutive ubiquitination in a Tyr(P)-1045-independent manner that is counteracted by UBPY-mediated de-ubiquitination.

Overexpression of Enzymatically Active UBPY Dub Domain Controls EGFR Degradation and Downstream Signaling to MAPK—To further define the requirements for complex formation of UBPY with the EGFR and UBPY-mediated regulation of EGFR ubiquitination, we made non-overlapping N-terminal (Dub) and C-terminal ("Rhodanese") UBPY deletion mutants (Fig. 1). In these mutants the catalytic activity is disconnected from the remainder of the molecule, which often leads to dominant negative behavior upon overexpression. Interestingly, overexpression of GFP-Dub and GFP-Dub-dSB in HEK293 cells induced an EGFR mobility shift that is typical for activated EGFRs (Fig. 7, *fifth panel*, *first* and *third lanes*). This shift was not seen when catalytically inactive GFP-Dub-C748A and GFP-Dub-dSB-C748A (*2nd* and *4th lanes*) or when GFP alone (*10th lane*) was expressed. An enlargement of this panel is supplied as online supplemental Fig. 1. Partial EGFR activation correlated with the ability of the catalytically active GFP-Dub and GFP-Dub-dSB constructs to strongly coprecipitate with the EGFR (Fig. 7, *third panel*), whereas their C748A mutant counterparts poorly associated with the EGFR. Most strikingly, overexpression of the GFP-Dub and GFP-Dub-dSB mutants was associated with the disappearance of intermediate EGFR proteolytic degradation products and moderately enhanced expression of the 180-kDa EGFR band compared with cells transfected with catalytic inactive C748A Dub mutants (Fig. 7, *second panel*). It is important to note that intermediate EGFR degradation products and decreased 180-kDa EGFR bands were also observed in EGFR-expressing cells containing GFP alone (Fig. 7, *10th lane*). When analyzing the ubiquitination status of the EGFR in cells co-expressing the Dub constructs, we found that EGFR ubiquitination was not significantly affected relative to GFP control (Fig. 7, *top panel*). Consistent with the (partial) activation of the EGFR, enhanced EGFR expression levels, and disappearance of intermediate EGFR degradation products, expression of catalytically active but not inactive GFP-Dub domains was associated with activation of both extracellular signal-regulated kinase 2 (Erk2) (p42) and Erk1 (p44) MAPK proteins under steady state conditions, *i.e.* in the absence of EGF (Fig. 7, *bottom panel*, compare the *1–4th lanes* with the *10th lane*).

To provide further evidence for the effect of the Dub domain on ligand-induced EGFR signaling and EGFR turnover, we generated three cell lines stably expressing protein G-tagged wild-type Dub (HER14-Dub-wt), catalytically inactive Dub-C748A (HER14-Dub-CA), and control empty vector (HER14-Vec). Consistent with the results obtained in HEK293 cells, the

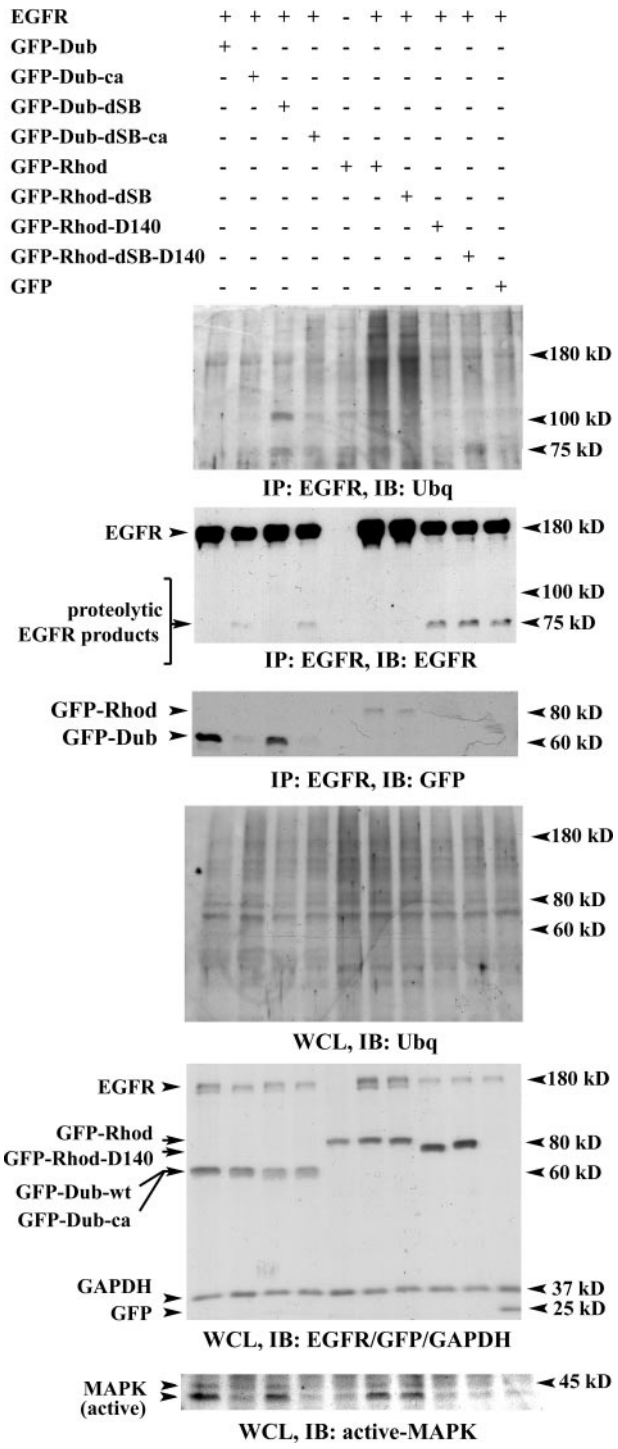

FIGURE 7. **UBPY affects constitutive EGFR turnover and downstream signaling to MAPK.** HEK293 cells were co-transfected with EGFR and GFP-tagged UBPY deletion constructs or pEGFP-c2 vector control (GFP) as indicated (see also Fig. 1). Serum-starved cells were lysed, and WCL were used for anti-EGFR IP and IB with anti-ubiquitin, anti-EGFR (Ab12), anti-GFP, anti-GAPDH, and anti-active MAPK as indicated. Data are representative of two experiments. *Ubq*, ubiquitin.

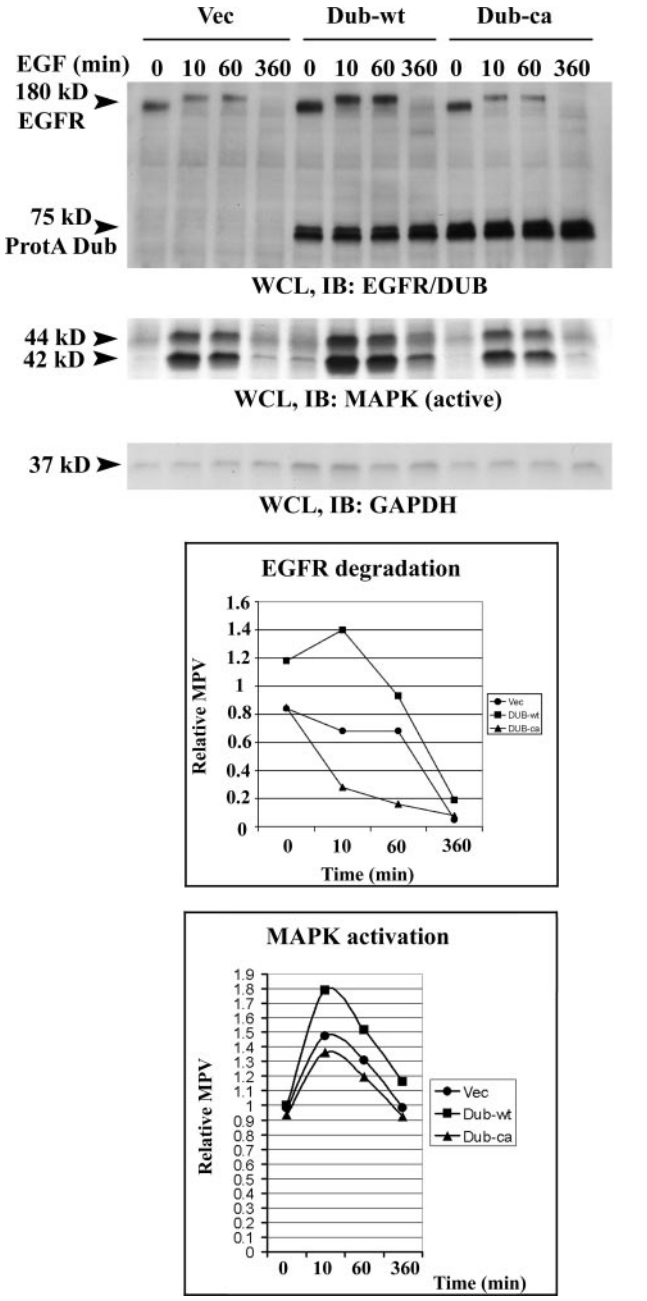

FIGURE 8. **UBPY affects ligand-induced EGFR turnover and downstream signaling to MAPK.** HER-14 cells that stably express protein A-tagged wild-type Dub domain, Dub-CA (C748A), or control vector (*Vec*) were generated. Serum-starved cells were stimulated for various time periods with EGF as indicated and lysed, and WCL were used for sequential IB with anti-EGFR, anti-active MAPK, and anti-GAPDH as indicated. The expressed protein A-tagged Dub domains were also detected by the IB procedure (*top panel*) due to protein A-IgG interaction. Expression levels of EGFR (*top panel*) and active MAPK (*middle panel*) were quantified and related to the GAPDH expression level (*bottom panel*) and plotted as a function of time (*bottom graphs*). Data are representative of two experiments. *MPV*, mean pixel value.

expression level of the EGFR in serum-starved HER14-Dubwt is already elevated compared with HER14-Dub-CA and HER14-Vec (Fig. 8, *top panel* and *top graph*) and remained so after EGF stimulation, although the EGFR was eventually degraded in all cell lines. Moreover, MAPK activation was markedly increased in wild-type Dub expressing cells even after 6 h of stimulation compared with HER14-Vec and HER14-Dub-CA (Figs. 8, *middle panel* and *bottom graph*). Collectively, these findings demonstrate that the C-terminal part of UBPY containing the Dub domain coprecipitates with the EGFR in an SB-C-independent and Cys-748-dependent manner with the EGFR. Overexpression of the catalytically active UBPY Dub domain leads to acti-

vation of the EGFR under steady state conditions that is associated with enhanced MAPK activation, moderately enhanced EGFR expression levels, and disappearance of intermediate EGFR degradation products.

Overexpression of the UBPY N-terminal Regulatory Domain Controls EGFR Ubiquitination, EGFR Degradation, and Downstream MAPK Signaling—We next evaluated whether overexpression of the N-terminal half-of UBPY, the Rhod construct, also affected EGFR turnover and downstream signaling. Importantly, co-expression of GFP-Rhod or GFP-Rhod-dSB with the EGFR strongly induced accumulation of ubiquitinated EGFRs, which migrate as a smear above the 180-kDa position on the blot (Fig. 7, *top panel*, compare the *5–10th lanes*). Interestingly, the smeared ubiquitin-signal also appeared well below the 180-kDa position on the blot, which may originate from ubiquitinated EGFR-binding proteins. Indeed, overexpression of GFP-Rhod induced accumulation of ubiquitinated cellular proteins (Fig. 7, *fourth panel*). Thus, in contrast to overexpression of the UBPY Dub domain, overexpression of the UBPY Rhod construct led to significant accumulation of ubiquitinated EGFRs as well as other cellular proteins, suggesting that this construct interfered in a dominant negative manner with endogenous UBPY de-ubiquitinating activity. The dominant negative activity of the UBPY Rhodanese construct did not require the N-terminal SH3 binding sequence but did require the N-terminal 140 amino acids of UBPY. The dominant-negative activity of the GFP-Rhod and GFP-Rhod-dSB constructs correlated (i) with their ability to coprecipitate with the EGFR (Fig. 7, *third panel*), (ii) with the disappearance of intermediate EGFR degradation products (Fig. 7, *second panel*), and (iii) with the accumulation of the 180-kDa EGFR (Fig. 7, *second panel*). In addition, as seen previously for the enzymatically active Dub domains, overexpression of the dominant negative GFP-Rhod and GFP-Rhod dSB constructs also led to the EGFR mobility shift that is characteristic for activated EGFR (Fig. 7, *fifth panel* and supplemental Fig. 1) as well as constitutive up-regulation of EGFR-dependent MAPK activation (Fig. 7, *bottom panel*, compare the *5–10th lanes*). Indeed, an EGFR doublet can be clearly observed upon coexpression of GFP-Rhod but not GFP-RhodD140 or GFP alone, although the mobility shift is somewhat less pronounced when compared with EGF stimulation (supplemental Fig. 2), consistent with partial activation of the EGFR. Moreover, as shown in supplemental Fig. 3 (*bottom panel*), the basal MAPK activation observed upon coexpression of EGFR with GFP Rhod but not GFP alone (compare the *lanes 1* and *5*) is clearly less than the MAPK activation observed upon EGF stimulation. It should be noted that HEK293 cells do contain small numbers of EGFRs, leading to significant EGF-induced MAPK activation in the absence of overexpressed EGFR. Collectively, these findings demonstrate that the N-terminal half of UBPY acts as a dominant negative and forms a complex with the EGFR in a manner that depends on the N-terminal 140 amino acids but not on the N-terminal Hbp SH3 binding motif (SB-N). Overexpression of the dominant negative N-terminal regulatory half of UBPY leads to enhanced activation of the EGFR under steady state conditions that is associated with enhanced EGFR ubiquitination, enhanced MAPK activation, enhanced EGFR expression levels, and disappearance of intermediate EGFR degradation products.

DISCUSSION

Here we demonstrate that the UBPY/Usp8 de-ubiquitinating enzyme is tyrosine-phosphorylated in response to EGF treatment in an EGFR- and Src-family kinase-dependent manner. Our data demonstrate that UBPY constitutively forms a complex with the EGFR and that catalytically inactive UBPY exhibits enhanced substrate binding (*i.e.* substrate-trap characteristics). Furthermore, UBPY de-ubiquitinates the EGFR *in vitro* and *in vivo* in a Cys-748-dependent manner. Non-overlapping N-terminal and C-terminal deletion constructs of UBPY act as dominant negatives *in vivo* by modulating constitutive and ligand-induced (i) EGFR ubiquitination, (ii) EGFR expression levels, (iii) appearance of intermediate EGFR degradation products, and (iv) downstream MAPK signaling.

Post-translational Modification of UBPY—Our findings indicate that UBPY is primarily a substrate for Src-family tyrosine kinases that are activated in response to EGFR ligand binding. Given that both UBPY (this study) and Src (31) coprecipitate with the EGFR, it is possible that UBPY is phosphorylated by Src in a trimolecular UBPY-EGFR-Src complex. Our findings indicate that the N-terminal but not the C-terminal half of UBPY is sufficient for Src-mediated tyrosine phosphorylation. However, it is formally possible that the Dub domain undergoes tyrosine phosphorylation within the context of the full-length UBPY. The functional relevance of UBPY tyrosine phosphorylation and the predicted recruitment of SH2 or PTB (phosphotyrosine binding) domain-containing proteins to UBPY remains unknown. Mapping of UBPY phosphorylation sites (in progress) will be necessary to determine its functional significance on UBPY de-ubiquitination activity, its subcellular distribution, its association with other proteins, and/or its substrate specificity *in vivo*.

As seen in Fig. 2, the GFP-Rhod and GFP-Dub constructs migrate as two separate bands on SDS-PAGE. Indeed, both endogenous as well as recombinantly expressed wild-type UBPY can be resolved into a doublet on SDS-PAGE. It is possible that this is due to post-translational modification, *e.g.* phosphorylation or ubiquitination. However, we have no reason to assume that the upper band is due to tyrosine phosphorylation of UBPY as the GFP-Rhod construct still migrates as a doublet in the presence of (i) EGFR kinase inhibitor PD153035 (Fig. 3*A*, *second panel*, *lanes 7* and *8*) or (ii) Src kinase inhibitor PP2 (Fig. 3*B*, *second panel*, *lanes 7* and *8*). In addition, the GFP-Dub domain is not tyrosine-phosphorylated, but it still migrates as a doublet (Fig. 2*C*). Furthermore, overlay of the blots (Fig. 2*C*, *first* and *second panels*; Fig. 3*A*, *first* and *second panels*; Fig. 3*B*, *first* and *second panels*) reveals that the prominent tyrosine-phosphorylated GFP-Rhod band corresponds to the lower band of the doublet. Prolonged exposures reveal that the upper band also contains phosphotyrosine (data not shown).

Although we do not have direct evidence for ubiquitination of wild-type or endogenous UBPY *in vivo*, inhibition of *in vitro* enzymatic activity of FLAG-tagged UBPY using the alkylating agent NEM not only leads to defects in de-ubiquitination of immunopurified ubiquitinated EGFR substrate but also to a

slight increase in the apparent molecular weight of wild-type FLAG-Usp8 on SDS-PAGE (data not shown), suggesting that immunopurified UBPY is ubiquitinated and undergoes NEM-sensitive auto-de-ubiquitination (25).

Complex Formation of UBPY with the EGFR in Vivo—Our findings demonstrate that UBPY constitutively forms a complex with the EGFR in a ligand- and EGFR kinase-independent manner, which contrasts with the EGF-induced EGFR-UBPY coprecipitation reported elsewhere (25). Moreover, the coprecipitation of the non-overlapping rhod (amino acids 1–504) and Dub constructs (amino acids (640–1080) with the EGFR points to a bivalent interaction of UBPY with the EGFR. Coprecipitation of the UBPY Rhod construct is dependent on the N-terminal 140 amino acids, whereas the coprecipitation of the UBPY Dub domain with the EGFR is dependent on the catalytic Cys-748 residue. A similar bivalent interaction involving the UBPY Rhodanese-like homology and Dub domains has recently been demonstrated between UBPY and its substrate Nrdp1, an E3 ubiquitin ligase for ErbB3 and ErbB4 proteins (32). It is possible that the interaction between UBPY and the EGFR is indirect, as pulldown experiments with GST-Rhod and GST-Dub domains and whole cell lysates obtained from unstimulated and EGF-stimulated HER-14 cells did not lead to co-purification of the EGFR (data not shown). Interaction of full-length UBPY with the EGFR increases profoundly when the catalytic cysteine is mutated, as has been reported by others (25). In contrast, the association of the truncated UBPY Dub domain with the EGFR is abolished by introduction of the C748A mutation, suggesting that the UBPY Dub domain recognizes the EGFR solely via active site binding of ubiquitin. Although the molecular basis for the apparent discrepant consequences of the C748A mutation in full-length UBPY as opposed to the isolated UBPY Dub domain is not known, it is possible that failure to de-ubiquitinate the EGFR by full-length UBPY C748A leads to prolonged complex formation through the UBPY N-terminal/Rhodanese region. Based on the crystal structure of HAUSP/Usp7 in the presence and absence of ubiquitin aldehyde (33) it is clear that DUB domains undergo a conformational change upon ubiquitin binding, which may prevent spurious binding of DUB enzymes to ubiquitinated cellular proteins. Selection of the proper substrate for specific Dub enzymes may be mediated by specialized substrate interactions domains outside the DUB domain, such as the UBPY Rhodanese homology domain or the N-terminal 140 amino acids of UBPY.

UBPY Affects EGFR Ubiquitination—Overexpression of either the catalytically inactive full-length UBPY or the truncated UBPY Rhod construct leads to enhanced constitutive and ligand-induced EGFR ubiquitination. In contrast, overexpression of the isolated UBPY Dub domain did not lead to obvious accumulation of ubiquitinated EGFRs. We currently favor the model that the isolated enzymatically active UBPY Dub domain prematurely de-ubiquitinates the EGFR at the plasma membrane or in an early endosomal compartment, thereby limiting ubiquitin-dependent EGFR sorting to the lysosomal pathway, which in turn could lead to enhanced MAPK signaling and reduced EGFR degradation (supplemental Fig. 4). In contrast, overexpression of the full-length UBPY C748A or C-terminally truncated Rhod construct leads to profound accumulation of ubiquitinated EGFRs as well as enhanced EGFR expression levels and MAPK activation. It is important to note that the effects of the dominant negative constructs on EGFR ubiquitination were still retained when the Hbp SH3 binding motifs were deleted (data not shown), indicating that the observed effects were not artifacts due to competition of these dominant negative UBPY constructs and AMSH for binding to the Hbp-Hrs complex. Although it seems counterintuitive that enhanced EGFR ubiquitination is correlated with enhanced EGFR expression levels, it seems possible that the full-length UBPY C748A and the UBPY Rhod construct are recruited to late endosomal membranes where they associate with evolutionary conserved ESCRT-III and/or Vps31/Alix proteins, thereby preventing recruitment of endogenous UBPY to late endosomal membranes. The failure to de-ubiquitinate EGFR cargo on late endosomal membranes may lead to reduced incorporation into internal vesicles of MVBs and consequent enhanced EGFR expression and downstream signaling (supplemental Fig. 4) (34). Consistent with our conclusions, Row and colleagues (26) recently reported prolonged retention times of the EGFR on late endosomes, enhanced endosomal ubiquitin staining, the appearance of MVBs with sparse internal vesicles, and large numbers of MVBs that were "stitched" together in UBPY knock-down cells. They speculated that these "stitched MVBs" could represent cargo proteins on the limiting membrane of MVBs that associate in *trans* with ubiquitin binding motifs on partner MVBs, and concluded that UBPY is essential for EGFR degradation (26). As mentioned earlier, Mizuno et al. (25) reached the conclusion that UBPY inhibits EGFR degradation. Although the underlying reason for the apparently conflicting data is presently unclear, we believe it is important to note that inhibition of UBPY function (using either RNA interference or dominant negative constructs) leads to enhanced EGFR ubiquitination and, thus, a shift to higher apparent molecular weights on Western blots, which confounds and, thus, limits the use of the 180-kDa EGFR band on Western blots as a quantitative measure for EGFR degradation.

Although we cannot formally exclude the possibility that the enhanced EGFR ubiquitination seen after overexpression of catalytically inactive full-length UBPY or the truncated Rhod construct is the result of enhanced recruitment of an E3 ligase, we favor the simplest model that enhanced EGFR ubiquitination is the result of impaired EGFR de-ubiquitination for a variety of reasons. First, we previously demonstrated that the EGFR undergoes de-ubiquitination (4). Second, enhanced EGFR ubiquitination induced by these dominant negative constructs correlates with their ability to coprecipitate with the EGFR. Third, it is not clear why the full-length UBPY C748A mutation would lead to a gain-of-function such as enhanced E3 ligase recruitment. The UBPY Rhod constructs show a very similar phenotype as the UBPY C748A mutant. Fourth, we[3] and others (32) have not been able to coprecipitate UBPY and the E3 ligase Cbl. Fifth, although it has previously been reported that UBPY can physically interact with Nrdp1, an E3 ligase for ErbB3 and ErbB4 proteins, disruption of UBPY enzymatic activity destabilizes the Nrdp1 protein by enhancing Nrdp1 ubiquitination and degradation (32). Sixth, a recent crystal structure of the UBPY N-terminal region (PDB code 2A9U) shows that this region can

dimerize. The requirement for the N-terminal region for the dominant negative activity of the UBPY Rhod construct may, therefore, be due to its ability to dimerize with endogenous UBPY proteins.

Given that overexpression of the UBPY Rhod construct leads to accumulation of ubiquitinated cellular proteins even in the absence of co-transfected EGFRs (Fig. 7), it is clear that the EGFR is not the only substrate for the de-ubiquitinating enzyme activity of UBPY (30, 32). We hypothesize that additional endosomal ubiquitinated cargo and/or endosomal adaptor proteins (*e.g.* Eps15, CIN85) may be substrates for UBPY. In yeast, the Doa4 ortholog has been shown to dynamically associate with proteasomes in sub-stoichiometric amounts (35). So far, UBPY has not been detected in mammalian proteasome preparations. However, if mammalian UBPY (transiently) associates with proteasomes, a model that is consistent with our previous findings of proteasome-dependent de-ubiquitination of the EGFR (4), then the number of potential substrates for UBPY may obviously be very large.

Our finding that dominant negative UBPY constructs dramatically enhance EGFR ubiquitination even in the absence of ligand provides novel and compelling evidence that steady state EGFR ubiquitination is determined by the balance between ubiquitination and de-ubiquitination under our assay conditions. Even more striking is our finding that dominant negative UBPY constructs enhance ubiquitination of EGFR wild-type and Y1045F constructs, which is in line with the finding by Huang *et al.* (12) that lysine residues within the kinase domain of EGFR Tyr-1045 mutants still undergo ubiquitination. Given the role of Doa4/Ubp4 in yeast, we hypothesize that such cycles of ubiquitination and de-ubiquitination occur at the plasma membrane or in an endosomal compartment. The dramatic ligand-independent EGFR ubiquitination seen in the presence of dominant negative UBPY Rhod constructs also raises the question of which E3 ubiquitin ligase is responsible for constitutive EGFR ubiquitination. Clearly, such ubiquitination is not mediated by Cbl recruitment to EGFR Tyr(P)-1045, although Cbl may also be recruited to the EGFR indirectly (6, 12, 15). Recently, SOCS proteins have been implicated in constitutive EGFR ubiquitination (36, 37), although the mechanism of SOCS recruitment to the EGFR is currently unclear. It is also possible that UBPY is not the only Dub enzyme that controls the ubiquitination status of the EGFR and other ErbB family members. Indeed, AMSH has recently been suggested to play a role in EGFR de-ubiquitination based on *in vitro* data (38).

In general, DUB enzymes may be involved in different biological processes such as (i) processing of tandem ubiquitin precursor chains, (i) recycling of ubiquitin from proteins that are targeted for proteasomal degradation, (iii) recycling of ubiquitin from proteins that are destined for vacuolar/lysosomal degradation, or (iv) trimming of poly-ubiquitin chains to mono/oligo-ubiquitin adducts (39). Recently, two groups reported that the EGFR is modified mostly by mono-ubiquitin adducts on multiple lysines rather than poly-ubiquitin chains (10, 11). More recently, using mass spectrometry, Huang et al. (12) reported that the EGFR is ubiquitinated on multiple lysines in the kinase domain and that a significant fraction of ubiquitin was present in the form of K63-linked poly-ubiquitin chains. The mechanism underlying Cbl-mediated mono- rather than poly-ubiquitination is, however, not clear. To reconcile these apparently conflicting data, we suggest the possibility that UBPY is involved in trimming Cbl-induced poly-ubiquitin chains to mono-ubiquitin adducts. Such a model would predict that the EGFR contains a mixture of mono- and poly-ubiquitin chains. Although it was originally reported that UBPY-mediated de-ubiquitination is specific for K48- as opposed to K63-linked poly-ubiquitin chains (38), more recent data from the same group suggest that UBPY can hydrolyze both K48- as well as K63-linked poly-ubiquitin linkages (26), which would be consistent with the model we propose.

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