

## Negative Regulation of HER2 Signaling by the PEST-type Protein-tyrosine Phosphatase BDP1\*

Received for publication, August 27, 2003, and in revised form, November 26, 2003  
Published, JBC Papers in Press, December 2, 2003, DOI 10.1074/jbc.M309527200

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**Signaling by receptor tyrosine kinases (RTK) mediates a variety of complex cellular functions and in case of deregulation can contribute to pathophysiological processes. A tight and finely tuned control of RTK activity is therefore critical for the cell. We investigated the role of the PEST-type protein-tyrosine phosphatase BDP1 in the regulation of HER2, a member of the epidermal growth factor receptor (EGFR) family of RTKs. Here we demonstrate that HER2 signaling is highly sensitive to BDP1 activity. Overexpression of BDP1 inhibited ligand-induced activation of HER2 but not that of the closely related EGFR. On the other hand, suppression of endogenous BDP1 expression increased the phosphorylation state of HER2. In addition, BDP1 was able to interfere with downstream signaling events by inhibiting the phosphorylation of the adaptor protein Gab1 and reducing mitogen-activated protein kinase activation. Supported by the finding that BDP1 is coexpressed with HER2 in breast cancer cells, we suggest that BDP1 is an important regulator of HER2 activity and thus the first protein-tyrosine phosphatase shown to be involved in HER2 signal attenuation.**

The epidermal growth factor receptor (EGFR)<sup>1</sup> family of receptor tyrosine kinases (RTKs) consists of the four closely related receptors EGFR (erbB1, HER1), HER2 (erbB2), HER3 (erbB3), and HER4 (erbB4). The common architecture of all EGFR family members comprises a ligand-binding extracellular domain, a transmembrane domain, and a cytoplasmic protein-tyrosine kinase domain. Activation of the receptors results from binding of a ligand of the EGF-like peptide growth factor family, which leads to the formation of receptor homo- and heterodimers (1). The subsequent activation of the intrinsic protein-tyrosine kinase triggers autophosphorylation of spe-

cific tyrosine residues within the cytoplasmic domain. These phosphorylation sites then serve as a docking platform for adaptor molecules like Grb2, Shc, or Gab1 and form the starting point for a variety of signaling cascades that regulate cell proliferation, differentiation, migration, and survival (2, 3).

Within the EGFR family of RTKs, HER2 has an outstanding role since it does not bind any known growth factor with high affinity. Instead of being activated by direct ligand binding, HER2 seems to be the preferred heterodimerization partner for other EGFR family members that define ligand specificity (4). Upon overexpression, HER2 also occurs as an active homodimer even in the absence of any ligand or heterodimerization partner. These constitutively active HER2 homodimers evade down-regulation mechanisms and cause transformation of immortalized cells (5, 6). According to this oncogenic potential, deregulation of HER2 as well as other EGFR family members is a hallmark of a variety of human cancers (7). For example, amplification of the *HER2* gene is found in 20–30% of early stage breast cancers and the consequential overexpression of the HER2 protein correlates with poor clinical prognosis and reduced survival of the patients (8, 9). Based on the discovery of the importance of aberrant HER2 activity in breast cancer, the monoclonal anti-HER2 antibody Trastuzumab (Herceptin) was developed representing the first genomics-based drug that was approved by the Food and Drug Administration for the treatment of women with HER2-overexpressing metastatic breast cancer (5, 11).

The wide range of normal cellular functions governed by EGFR family members and the pathophysiological significance of unrestrained kinase activity underscore the requirement of a tight and finely tuned regulation of RTK signaling. Various mechanisms have been shown to act in concert in order to ensure an appropriate duration and magnitude of receptor signaling, which will ultimately determine cell fate. In the case of the EGFR, several ways of receptor down-regulation have been identified (12). These include ligand-induced endocytosis (13), degradation by the ubiquitin-proteasome machinery (14, 15), as well as transcriptional induction of inhibitory proteins that counteract downstream signaling (16–18). However, it is clear that an important way of regulating receptor activity is the control of its phosphorylation state. Several protein-tyrosine phosphatases (PTPs) were shown to be able to counteract EGFR activity by abrogating receptor autophosphorylation and thereby blocking downstream signaling (19, 20). For example, PTP1B was implicated in EGFR regulation in an overexpression study by Lammers *et al.* (21). A recent report (22) confirming these findings showed that in fibroblasts from PTP1B-deficient mice, the phosphorylation of the EGFR is increased and sustained after growth factor treatment. Another example for an EGFR-regulating PTP is the receptor-like phosphatase RPTP $\sigma$ , which was reported to impair EGFR activation, substrate phosphorylation, and signaling (23).

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<sup>1</sup> The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; BDP1, brain-derived phosphatase 1; C/S, catalytic cysteine to serine mutated; ERK, extracellular signal-regulated kinase; Gab1, Grb2-associated binder 1; GST, glutathione S-transferase; HA, hemagglutinin; HEK, human embryonal kidney; HER2, human epidermal growth factor receptor 2; IGF1R, insulin-like growth factor receptor; IP, immunoprecipitation; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMTV, mouse mammary tumor virus; PEST, proline, glutamic acid, serine and threonine; PTP, protein-tyrosine phosphatase; RTK, receptor tyrosine kinase; WAP, whey acidic protein; siRNA, small interfering RNA.

Although the biology and pathology of HER2 has been under extensive investigation since its discovery in 1984 (24, 25), no PTP has been implicated in the regulation of the HER2 receptor so far. In our effort to identify new substrates of PTPs, we found that the protein-tyrosine phosphatase BDP1 might be involved in the control of HER2 activity. BDP1 (also known as PTP20 (26), PTP-HSCF (27), PTP-K1 (28), or FLP1 (29)) belongs to the family of PEST-containing PTPs, which also includes PTP-PEST and PEP (30, 31). BDP1, PTP-PEST, and PEP share a common structure composed of a N-terminal catalytic domain and a non-catalytic C-terminal sequence that comprises the eponymous proline-, glutamic acid-, serine-, and threonine-enriched PEST sequences. In earlier publications (32, 33), BDP1 has already been implicated in the regulation of tyrosine kinases. Here we show that BDP1 is able to negatively affect HER2 activation as well as downstream signaling events. Furthermore, BDP1 is shown to be coexpressed with HER2 in breast cancer cell lines. Taken together, these results point toward BDP1 as an important negative regulator of HER2-mediated signaling and therefore support a role of this PTP as a tumor suppressor.

#### EXPERIMENTAL PROCEDURES

**Antibodies and Reagents**—For generation of BDP1 antibody, cDNA encoding amino acids 295–436 of BDP1 were cloned into a pGEX vector (Amersham Biosciences). The GST fusion protein was expressed in *Escherichia coli*, purified, and used to immunize rabbits. A monoclonal anti-HER2 antibody was used for immunoprecipitation and the polyclonal anti-HER2-CT for Western blot analysis (34). The monoclonal HER2 blocking antibody 2C4 was described previously (35). EGFR was precipitated with monoclonal antibody anti-EGFR 108.1 (36) and detected by Western blot using sheep polyclonal antibody anti-EGF receptor (Upstate Biotechnology, Inc.). Monoclonal antibody anti-IGF1R- (24–31) (37) and polyclonal anti-Shc antibodies (38) have been described before. Anti-Gab1 (H-198) was from Santa Cruz Biotechnology, and anti-hemagglutinin (HA.11) was from Babco. Phosphorylation state-specific antibodies for ERK2 and JNK were from New England Biolabs. Phosphotyrosines were detected with the 4G10 monoclonal antibody (Upstate Biotechnology, Inc.). Horseradish peroxidase-conjugated goat anti-rabbit antibody was from Bio-Rad; goat anti-mouse antibody was from Sigma, and rabbit anti-sheep was from Dianova. Chemiluminescence reagents were from PerkinElmer Life Sciences. Protein A-Sepharose beads were purchased from Amersham Biosciences. EGF was supplied by Invitrogen and heregulin- $\beta$ 1 and IGF1 by Sigma. The nitrocellulose membrane was from Schleicher & Schüll. All of the other reagents were obtained from Merck.

**Plasmid Construction and Protein Purification**—The expression construct for wild type BDP1 was described previously (32). The catalytically impaired C/S mutant of BDP1 was obtained by site-directed mutagenesis replacing cysteine 229 by serine and the substrate trapping D/A mutant by replacing aspartic acid 197 by alanine. N-terminally HA-tagged BDP1 and HE-PTP were generated by PCR. GST fusion constructs were obtained by cloning wild type BDP1, BDP1 C/S, and BDP1 D/A, respectively, into the pGEX5x vector (Amersham Biosciences) in-frame with the GST gene. All of the GST fusion proteins were expressed in the BL21 DE3 codon+ (Stratagene) and purified with GSH-Sepharose beads. To target BDP1 protein for down-regulation with siRNA, we generated the pSuperRetro-BDP1 (pSR-BDP1) construct according to supplier's instructions (OligoEngine). The 19-nucleotide BDP1 target sequences was GAGGGACACAGCGACTACA.

**Cell Culture and Transfection**—All cell lines were obtained from either the German or American Tissue Culture Collection DSMZ and ATCC, respectively, and cultivated following the supplier's instructions. The cell culture reagents were purchased from Invitrogen. HEK 293 cells were transfected with 2  $\mu$ g of DNA/ml of medium by the calcium phosphate precipitation method (39). After 24 h, the cells were transferred to serum starvation medium and cultured for another 24 h before stimulation and lysis.

**Retroviral Infection**—Amphotropic retroviral supernatants were produced by transfection of phoenix packaging cells (kindly provided by G. Nolan, Stanford) by the calcium phosphate precipitation method (40). 48 h post-transfection, the tissue culture medium was filtered through a 0.45- $\mu$ m filter, and the viral supernatant was used for infection of

T47D cells after addition of 4  $\mu$ g/ml Polybrene. Cells were infected three times for at least 4 h and allowed to recover for 24 h with fresh medium. Cells were starved in serum-free medium 24 h before stimulation and lysis.

**Preparation of Tumor Lysates**—Breast tumors from transgenic MMTV-HER2 (41), WAP-TGF $\alpha$ /Wnt (42), MMTV-PMT (43), MMTV-Wnt (44), and WAP-SV40-TAg (45) mice were kindly provided by Lothar Hennighausen (National Institutes of Health, Bethesda). Tissues were homogenized in lysis buffer (20 mM Tris/HCl, pH 8.0, 140 mM NaCl, 10% glycerol, 2 mM EDTA, and 1% Nonidet P-40) supplemented with phosphatase and protease inhibitors (10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 1 mM NaF, and 0.5% aprotinin).

**Immunoprecipitation and Western Blot**—The cell cultures were washed with PBS and lysed with lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerine, and 1% Triton X-100) supplemented with phosphatase and protease inhibitors (10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 1 mM NaF, and 0.5% aprotinin). The cellular debris was removed by centrifugation. For immunoprecipitations, whole cell lysates were combined with antibody and 30  $\mu$ l of protein A-Sepharose. The samples were incubated for 3 h on a rotation wheel at 4 °C. The precipitates were washed three times with 0.5 ml of HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerin, and 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>), suspended in 3 $\times$  SDS sample buffer, boiled for 3 min, and subjected to SDS-PAGE. For Western blot analysis, the proteins were transferred to nitrocellulose membranes and incubated with the appropriate antibodies. If quantification was necessary, the filters were exposed to the LAS1000 chemiluminescence camera (Fujifilm) and analyzed with the program ImageGauge 3.3 (Fujifilm).

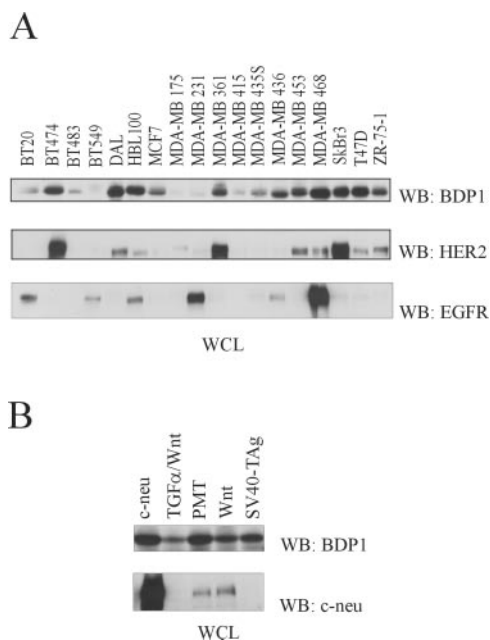
**In Vitro Binding Study**—Prior to lysis, SkBr3 cells were treated with 50 mM pervanadate for 20 min. Cells were rinsed with phosphate-buffered saline and lysed in 1% Nonidet P-40 buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM HEPES, pH 7.5, 1 mM EDTA, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml leupeptin, 1 mM benzamide). Additionally, the lysis buffer also contained 5 mM iodoacetic acid to irreversibly inhibit cellular PTPs. After incubation on ice for 5 min, dithiothreitol was added to a final concentration of 10 mM to inactivate excess iodoacetic acid. Insoluble debris was removed by centrifugation. Cell lysates were incubated with 100 ng of fusion protein of GST and wild type BDP1, the catalytically inactive C/S mutant, or the substrate-trapping D/A mutant. After addition of 20  $\mu$ l of GSH-Sepharose beads, the lysates were incubated at 4 °C for 2 h and washed three times with 1% Nonidet P-40 buffer. Samples were dissolved in 3 $\times$  SDS sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting.

#### RESULTS

**Expression Profile of BDP1, HER2, and EGFR in Breast Cancer Cell Lines and Mouse Mammary Tumors**—To address the question whether BDP1 might be involved in the negative regulation of EGFR family kinase signaling, we examined 17 breast cancer cell lines and 1 normal breast epithelial cell line for BDP1 as well as HER2 and EGFR expression. To this end, crude cell lysates were analyzed by immunoblotting with the appropriate antibodies. Fig. 1A shows that all cell lines expressing HER2 also show expression of BDP1. In particular BT474 and SkBr3 breast cancer cells, which are known to overexpress HER2 due to gene amplification (46), also contain high amounts of the phosphatase. On the other hand, in most cell lines with low or even no detectable HER2 expression, BDP1 levels were also substantially lower. In contrast to HER2, the protein levels of the EGFR did not correlate well with the expression of BDP1.

Next we compared the protein level of BDP1 in breast tumors from transgenic mice expressing HER2 or other oncogenes under the control of gene promoters, which restrict oncogene expression to the mammary alveolar epithelium (41–45). Although BDP1 was readily detected in all samples tested, its expression was maximal in the HER2-overexpressing tumor (Fig. 1B).

**BDP1 Overexpression Reduces HER2 Phosphorylation in HEK 293 Cells**—To investigate whether BDP1 would be able to interfere with HER2-mediated signaling, we examined the impact of BDP1 overexpression on the phosphorylation of the



**FIG. 1. Expression levels of BDP1, HER2, and EGFR in human mammary cell lines and mouse mammary tumors.** A, cell lines were cultured under normal conditions. After lysis, 50  $\mu$ g of each whole cell lysate (WCL) was resolved by SDS-PAGE, followed by Western blot (WB) analysis using anti-BDP1, anti-HER2, and anti-EGFR antibodies. B, whole cell lysates (50  $\mu$ g) of mammary tumors from transgenic mice carrying the indicated oncogene under the control of a mammary-specific promoter (MMTV-HER2, WAP-TGF $\alpha$ /Wnt, MMTV-PMT, MMTV-Wnt, and WAP-SV40-TAg) were analyzed by anti-BDP1 and anti-HER2 immunoblotting.

endogenous HER2 receptor in HEK 293 cells. In control cells transfected with empty vector, tyrosine phosphorylation of HER2 was rapidly induced by stimulation with EGF, reaching its maximum within 1 min (Fig. 2, A and B). In cells overexpressing BDP1, activation of HER2 was strongly reduced and also slightly retarded. The effect of BDP1 on HER2 phosphorylation was dependent on its enzymatic phosphatase activity, because exogenous expression of a catalytically inactive C/S mutant did not counteract the phosphorylation of HER2 (Fig. 2C). In contrast, a slight increase in HER2 phosphorylation could be observed. Positive effects of C/S mutants of phosphatases have been reported before (33) and likely reflect a competition with endogenous phosphatases resulting in “dominant-positive” effects. Whereas activation of HER2 by EGF stimulation is triggered by heterodimer formation of HER2 with the EGFR, we next used heregulin- $\beta$ 1 to induce and activate HER2-HER3 dimers. As demonstrated in Fig. 2D, exogenous expression of the phosphatase diminished HER2 activation after heregulin- $\beta$ 1 stimulation as well. This indicated that the ability of BDP1 to impair HER2 phosphorylation is independent of the HER2 binding partner in the dimeric receptor complex.

The potential of exogenous BDP1 to diminish the phosphorylation of HER2 raised the question as to whether HER2 and BDP1 would directly interact. To address this point, we performed an *in vitro* binding study using purified fusion proteins of GST and either the wild type form, the catalytically inactive C/S mutant, or the substrate-trapping D/A mutant form of BDP1. HER2-overexpressing SkBr3 breast cancer cells were treated with the unspecific phosphatase inhibitor pervanadate to induce maximal cellular phosphorylation and subsequently lysed. As Fig. 2E shows, the substrate-trapping mutant of BDP1 precipitated a single tyrosine-phosphorylated protein, which could be identified as HER2 by immunoblotting.

**Negative Regulatory Effect of BDP1 Is Specific for HER2 in HEK 293 Cells**—Because HER2 belongs to the family of EGFR-related receptors, we investigated whether BDP1 expression generally counteracts the signaling of RTKs of the EGFR family or if this effect is specific for the HER2 receptor. For this reason, the activation level of the EGFR was analyzed in HEK 293 cells transfected with BDP1 constructs. In contrast to the strong reduction of HER2 phosphorylation (Fig. 2), EGF-mediated EGFR activation was only marginally affected by overexpression of wild type BDP1 or the inactive C/S mutant (Fig. 3A). Likewise, phosphorylation of the unrelated RTK insulin-like growth factor 1 receptor (IGF1R) upon stimulation with its ligand IGF1 was not affected by the presence of exogenous BDP1 (Fig. 3B).

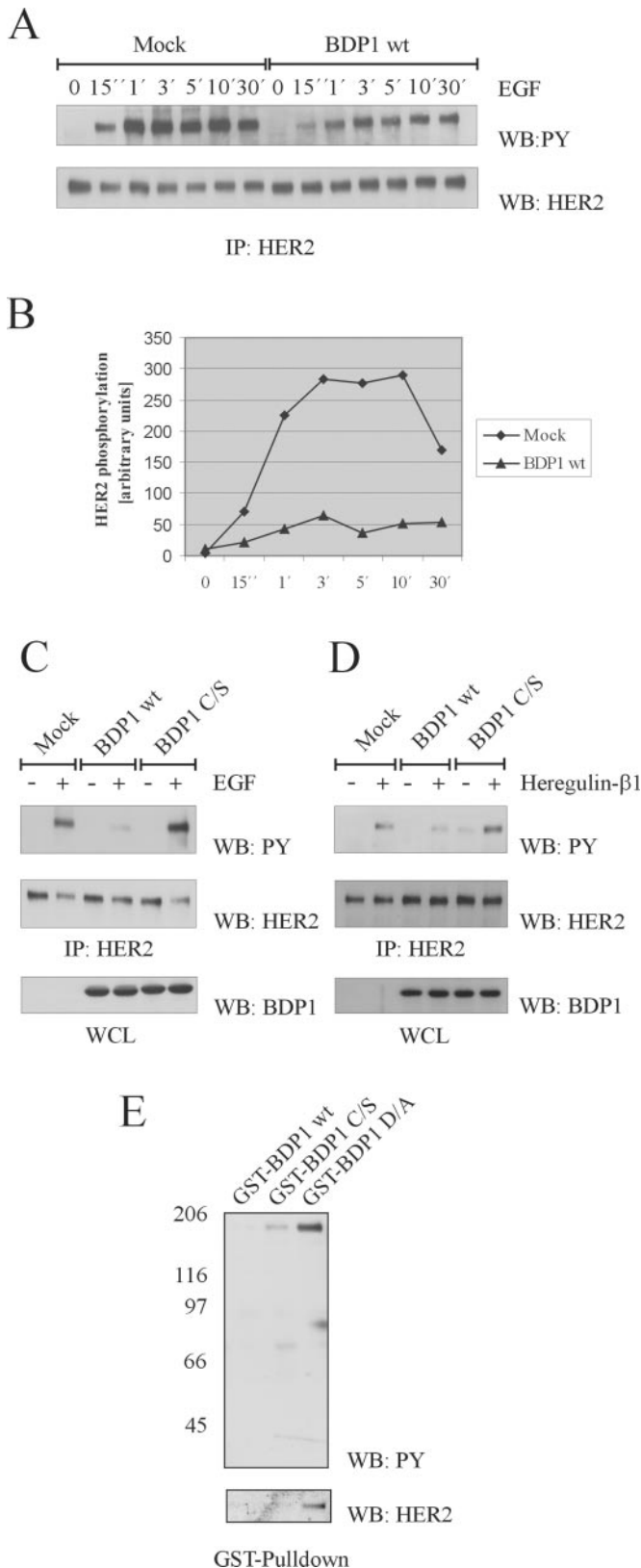
The specificity of BDP1 for HER2 was further exemplified by the observation that overexpression of the unrelated cytoplasmic phosphatase HePTP did not influence EGF-induced HER2 activation at all (Fig. 3C).

**Overexpression of BDP1 Interferes with EGF-mediated Signaling Events**—Activated RTKs recruit and phosphorylate a set of adaptor proteins, thereby initiating signaling cascades leading to specific cellular responses (47). To test whether BDP1 would be able to affect downstream events of HER2, we analyzed the EGF-induced phosphorylation of Shc and Gab1 adaptor proteins in the presence and absence of exogenous BDP1 in HEK 293 cells. Whereas neither the 46- nor the 52-kDa isoform of Shc was affected by overexpression of BDP1 (Fig. 4A), phosphorylation of Gab1 was almost completely abolished (Fig. 4B). A longer exposure of the same immunoblot further showed that the C/S mutant form of BDP1 had the potential to enhance the phosphorylation of Gab1 in unstimulated cells (data not shown).

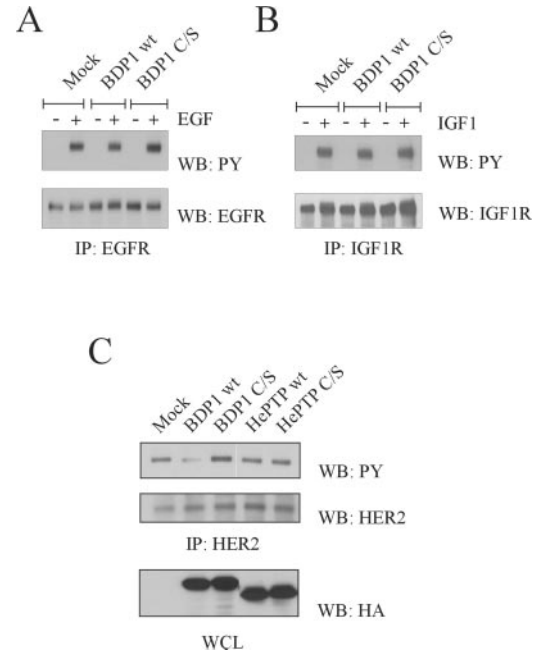
Recently, Gab1 has been implicated in EGF-induced activation of mitogen-activated protein kinases (MAPK) (48). Therefore, we examined if the activation of ERK2 and JNK1, two members of the MAPK family, would be perturbed in cells overexpressing BDP1. MAPK activity was assessed by immunoblotting using antibodies that specifically recognize the activated form of the respective MAPK. As shown in Fig. 4, C and D, EGF stimulation of cells resulted in a strong increase of ERK2 activity and a less pronounced JNK1 activation, respectively. Activation of both kinases was reduced to 50% in cells expressing wild type BDP1. The catalytically impaired C/S mutant of the phosphatase did not affect the activation of either MAPK.

To test whether the lack of Gab1 and MAPK phosphorylation in BDP1-overexpressing cells treated with EGF reflects an event downstream of HER2 as opposed to the EGFR, we inhibited HER2 activation by an alternative mechanism. Prior stimulation, cells were preincubated with the monoclonal HER2-specific blocking antibody 2C4 (35, 49). As shown in Fig. 4E, pretreatment of HEK 293 cells with 2C4 strongly reduced EGF-induced phosphorylation of HER2 and Gab1, whereas Shc phosphorylation was not affected. Similar to exogenous expression of BDP1, inhibition of HER2 by the blocking antibody resulted in an decrease of EGF-induced ERK1/2 activity. Taken together, these results show that BDP1 is not only able to decrease phosphorylation of HER2 but also its downstream signaling events.

**Down-regulation of Endogenous BDP1 by siRNA Enhances Her2 Phosphorylation in T47D Breast Cancer Cells**—By having established that increased BDP1 activity, in terms of overexpression, results in a reduction of HER2 signaling, we used an RNA interference approach to conduct the converse experiment. The level of endogenous BDP1 in T47D breast cancer cells was reduced by infection with a retroviral expression



**FIG. 2. Overexpression of BDP1 reduces HER2 phosphorylation in HEK 293 cells.** *A*, HEK 293 cells were transiently transfected with pcDNA3 BDP1 wild type (*wt*) or empty vector (*Mock*) and starved for 24 h. After stimulation with 5 ng/ml EGF, cells were lysed at the indicated time points. Anti-HER2 immunoprecipitations (*IP*) were analyzed using anti-phosphotyrosine (*PY*) and anti-HER2 antibodies. *WB*, Western blot. *B*, the phosphorylation level of HER2 in *A* was quantified by densitometry. The HER2 phosphorylation in untreated cells transfected with the empty vector was set as 1. *C* and *D*, HEK 293 cells transfected with pcDNA3, pcDNA3 BDP1 wild type, or pcDNA3 BDP1



**FIG. 3. Down-regulating effect of BDP1 is specific for HER2.** *A* and *B*, HEK 293 cells were either transfected with empty vector control (*Mock*) or with expression constructs of wild type BDP1 (*wt*) or the C/S mutant and starved for 24 h. *A*, after stimulation with 5 ng/ml EGF for 5 min, cells were lysed, and anti-EGFR immunoprecipitates (*IP*) were analyzed by Western blot (*WB*) using anti-phosphotyrosine (*PY*) and anti-EGFR antibodies. *B*, cells were stimulated with 25 ng/ml IGF1 for 5 min and were lysed. Anti-IGF1R immunoprecipitates were analyzed by anti-phosphotyrosine and anti-IGF1R Western blotting. *C*, transfected HEK 293 cells expressing HA-tagged forms of either the wild type or the inactive C/S mutant of BDP1 or the unrelated phosphatase HePTP were stimulated with 5 ng/ml EGF for 5 min and lysed. Anti-HER2 immunoprecipitates were analyzed by anti-phosphotyrosine and anti-HER2 Western blotting. Whole cell lysates (*WCL*) were immunoblotted with anti-HA antibody.

construct for a BDP1-specific small interfering RNA (Fig. 5). The cells were then stimulated with heregulin-β1, and the level of HER2 activation was examined. In accordance with the experiments using the overexpressed phosphatase (Fig. 2), a decrease of the endogenous BDP1 protein level reproducibly leads to an apparent increase of HER2 phosphorylation, which further supports the proposed role of BDP1 in the regulation of HER2-mediated signaling.

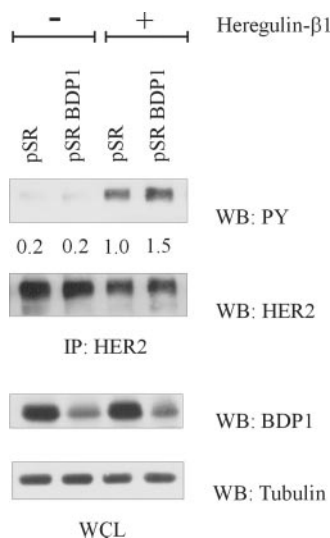
#### DISCUSSION

Signaling through RTKs is a major mechanism for intercellular communication during development and in the adult organism as well as in disease-associated processes (47). The phosphorylation status and signaling activity of RTKs is determined not only by the kinase activity of the RTK but equally by the activities of negatively regulating PTPs. Since their discovery in 1988 (50, 51), a tumor suppressor function of PTPs has been postulated based on their potential to counteract oncogenic kinase signaling. Despite extensive investigations, only

C/S were stimulated with 5 ng/ml EGF (*C*) or 25 ng/ml heregulin-β1 (*D*) for 5 min. Anti-HER2 immunoprecipitates were analyzed as in *A*. Expression of BDP1 forms is shown by immunoblot analysis of 30 μg of whole cell lysate (*WCL*) using anti-BDP1 antibody. *E*, SkBr3 cells were stimulated with 50 mM pervanadate for 20 min and lysed. *In vitro* binding studies were performed using GST fusion proteins of wild type BDP1 (GST-BDP1 *wt*), the catalytically inactive C/S mutant (GST-BDP1 C/S), and the substrate trapping D/A mutant (GST-BDP1 D/A). After precipitation with GSH-Sepharose, complexes were subjected to SDS-PAGE and analyzed using anti-phosphotyrosine (*PY*) and anti-HER2 antibodies.



**FIG. 4. Effect of BDP1 overexpression on EGF-mediated downstream signaling in HEK 293 cells.** A–D, HEK 293 cells were transfected with expression constructs of BDP1 wild type (*wt*), C/S mutant, or empty vector (*Mock*) and starved by serum deprivation for 24 h. A and B, after stimulation with 5 ng/ml EGF for 5 min, cells were lysed and anti-Shc (A), and anti-Gab1 (B) immunoprecipitates (IP) were analyzed by Western blot (WB) using anti-phosphotyrosine (PY), anti-Shc, or anti-Gab1 antibodies. C and D, cells were stimulated with 50 ng/ml EGF for 20 min, and whole cell lysates were analyzed. The levels of activated ERK2 and JNK1 were detected by immunoblotting with the respective phosphorylation state-specific antibodies and quantified by densitometry. The activity of each kinase in untreated cells transfected with the empty vector was set as 1-fold. The data represent the mean of three independent experiments  $\pm$  S.D. E, cells were starved for 24 h and preincubated with 56  $\mu$ M anti-HER2 antibody 2C4 for 70 min. After stimulation with 5 ng/ml EGF for 5 min, cells were lysed, and anti-HER2, anti-Gab1, and anti-Shc immunoprecipitates were analyzed by Western blot (WB) using anti-phosphotyrosine (PY), anti-HER2, anti-Shc, or anti-Gab1 antibodies. 30  $\mu$ g of whole cell lysate (WCL) were analyzed using a phosphorylation state-specific anti-phospho-ERK1/2 (P-ERK1/2) antibody.



**FIG. 5. Suppression of BDP1 expression by siRNA increases HER2 phosphorylation in T47D cells.** T47D breast cancer cells were either infected with retroviral pSuperRetro (pSR) BDP1 generating BDP1-specific small interfering RNAs or the empty control vector. After serum starvation for 24 h, cells were stimulated with 20 ng/ml heregulin- $\beta$ 1 for 5 min and lysed. Anti-HER2 immunoprecipitates (IP) were analyzed by immunoblotting with anti-phosphotyrosine (PY) and anti-HER2 antibodies and quantified by densitometry. The numbers below the lanes represent the relative phosphorylation level of HER2 (upper panels). Endogenous BDP1 levels were assessed by immunoblotting of whole cell lysates (WCL) with anti-BDP1 antibody. Equal loading was confirmed by anti-tubulin immunoblotting (lower panels).

little evidence has emerged that supports that hypothesis. A recent publication by Jandt *et al.* (52), for example, relates the ability of DEP-1 to modulate growth factor-stimulated cell migration and cell-matrix adhesion to the function of DEP-1 as a tumor suppressor.

To date, several phosphatases have been implicated in the negative regulation of EGFR activity and its downstream signaling. Among these are the receptor phosphatases RPTP $\sigma$  and LAR (23, 53) as well as the cytoplasmic phosphatases SHP-1, PTP1B, and TCPTP (54–56). In contrast, no phosphatase responsible for the inactivation of HER2 had been identified so far. Here we report that the protein-tyrosine phosphatase BDP-1 is able to regulate HER2 signaling. The phosphorylation and signaling potential of HER2 was highly sensitive to the catalytic activity of BDP1. Enhancing BDP1 activity in terms of overexpression potently diminished inducible HER2 activity. On the other hand, reducing the level of BDP1 in T47D breast cancer cells with siRNAs resulted in increased HER2 phosphorylation. These observations suggest that BDP1 is responsible for the attenuation of ligand-induced HER2 signal. *In vivo*, however, the regulation of HER2 can be expected to be much more complex as is the case for its closest homologue the EGFR. It is thus conceivable that rather than BDP1 alone numerous phosphatases act in concert in order to provide a tight and finely tuned regulation of the HER2 signal.

PTPs are highly specific enzymes dephosphorylating very selective phosphotyrosine proteins (57). The specificity of BDP1 for HER2 is exemplified by its inability to counteract neither EGFR nor IGFR phosphorylation. Nevertheless, a minor decrease in EGFR phosphorylation was occasionally observed in the presence of exogenous BDP1. This might reflect the indirect influence of BDP1 on the particular fraction of EGFRs that is engaged in HER2-EGFR heterodimers and is thus transphosphorylated to a lesser extent by the inhibited binding partner.

A consequence of impaired RTK activation is the failure to induce the early events of downstream signaling cascades, namely recruitment and phosphorylation of signaling mole-

cules (58). The adaptor protein Shc has been shown to bind to and to be phosphorylated by activated HER2 (59) and to play a role in HER2-mediated tumorigenesis (60). In HEK 293 cells, overexpression of BDP1 did not affect Shc phosphorylation after EGF stimulation. This observation suggests that in this system, EGF-induced Shc phosphorylation does predominantly occur downstream of the EGFR, which was not influenced by exogenously expressed BDP1. In contrast to Shc, phosphorylation of Gab1 was severely reduced by inhibition of HER2 activation by overexpression of BDP1 or by treatment of cells with the specific blocking antibody 2C4. Although direct binding of Gab1 to and phosphorylation by HER2 has not been shown so far, recent reports described the identification of Gab1 as a downstream effector of HER2 (61) and provided evidence for the requirement of Gab1 for HER2-driven transformation (62). Our findings suggest that upon stimulation of HEK 293 cells with EGF, Gab1 is mainly activated downstream of HER2-containing heterodimers, whose inhibition is reflected in impaired Gab1 phosphorylation.

After dimerization and subsequent phosphorylation, RTKs like HER2 and the EGFR induce the activation of MAPKs, which transduce the signal to the nucleus and there regulate the expression of effector genes (63). The best known pathway leading to the activation of MAPKs involves the Shc adaptor protein, recruitment of the Grb2/Sos complex, and activation of the small GTPase Ras (10). In addition to this Shc-involving pathway, Gab1 was identified as another signal transducer linking RTKs to MAPK activation (48, 62). We have shown that BDP1 was able to reduce the activation of two MAPKs, ERK2 and JNK, by about 50%. Having demonstrated before that BDP1 blocks Gab1 but not Shc phosphorylation, this partial reduction of MAPK activity presumably reflects the loss of signal transduction through Gab1 leaving only the Shc-dependent pathway intact.

We could show that BDP1 down-regulates the phosphorylation of HER2 protein itself as well as downstream signaling events, but how this is accomplished by BDP1 remains elusive. In light of the fact that BDP1 was able to bind to HER2, the most obvious explanation would be that BDP1 could be recruited to activated receptor complexes and that phosphorylated HER2 would be a direct substrate for BDP1.

Although the demonstrated reduction of Gab1 phosphorylation is most likely a consequence of HER2 being dephosphorylated and inactivated, we cannot exclude the possibility that Gab1 itself might serve as a substrate for BDP1 as well. Interestingly, Gab1 mediates a positive feedback loop that was shown to enhance the initial signal from activated EGFR (48). If Gab1 possesses a similar function in HER2 signaling, BDP1 might be able to regulate or even to abrogate such a positive feedback loop by controlling the phosphorylation state of Gab1. The question whether BDP1 would be able to influence the tumorigenic potential of HER2 *in vivo* remains to be addressed by ongoing research.

**Acknowledgments**—We thank Dr. Lothar Hennighausen for helpful discussions and for providing the mouse tumor samples. We also thank Dr. Yeon Woong Kim for providing BDP1 expression constructs.

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