Activation of Nonreceptor Tyrosine Kinase Bmx/Etk Mediated by Phosphoinositide 3-Kinase, Epidermal Growth Factor Receptor, and ErbB3 in Prostate Cancer Cells*

Received for publication, April 24, 2007, and in revised form, August 28, 2007 Published, JBC Papers in Press, September 6, 2007, DOI 10.1074/jbc.M703412200

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Pathways activated downstream of constitutively active phosphatidylinositol (PI) 3-kinase in PTEN-deficient prostate cancer (PCa) cells are possible therapeutic targets. We found that the nonreceptor Tec family tyrosine kinase Bmx/Etk was activated by tyrosine phosphorylation downstream of Src and PI 3-kinase in PTEN-deficient LNCaP and PC3 PCa cells and that Bmx down-regulation by short interfering RNA markedly inhibited LNCaP cell growth. Bmx also associated with ErbB3 in LNCaP cells, and heregulin-β1 enhanced this interaction and further stimulated Bmx activity. Epidermal growth factor (EGF) similarly stimulated an interaction between Bmx and EGF receptor and rapidly increased Bmx kinase activity. Bmx stimulation in response to heregulin-β1 and EGF was Src-dependent, and heregulin-β1 stimulation of Bmx was also PI 3-kinase-dependent. In contrast, the rapid tyrosine phosphorylation and activation of Bmx in response to EGF was PI 3-kinase-independent. Taken together, these results demonstrate that Bmx is a critical downstream target of the constitutively active PI 3-kinase in PTEN-deficient PCa cells and further show that Bmx is recruited by the EGF receptor and ErbB3 and activated in response to their respective ligands. Therefore, Bmx may be a valuable therapeutic target in PCa and other epithelial malignancies in which PI 3-kinase or EGF receptor family pathways are activated.

The class I PI3 kinase family produces phosphatidylinositol 3,4,5-triphosphate (PIP3), which recruits proteins containing PIP3-binding pleckstrin homology (PH) domains to the membrane. This PI3-kinase pathway is negatively regulated by PTEN, which functions as a PIP3 phosphatase. PTEN loss and subsequent activation of PI3-kinase signaling makes a major contribution to prostate cancer (PCa), with a large fraction of metastatic PCa and many high grade primary PCa being PTEN-deficient (2). Studies in mice have confirmed that lack of PTEN promotes the development of PCa, as mice completely lacking PTEN expression in the prostate develop high grade prostate intraepithelial neoplasia (PIN) at early ages and progress to adenocarcinoma (3–5). The serine/threonine protein kinase Akt is the best studied target of this PI3-kinase pathway, with Akt functioning to inactivate a number of growth-suppressing or pro-apoptotic substrates (6). Prostate-specific expression of a constitutively activated membrane-targeted Akt causes lesions that resemble PIN, but these lesions do not progress to adenocarcinoma, indicating that additional proteins downstream of PTEN loss and PI3-kinase pathway activation contribute to PCa growth (7).

One such candidate downstream target of PI3-kinase is Bmx (also termed Etk), a nonreceptor tyrosine kinase member of the Tec kinase family (8). Tec kinases are unique among tyrosine kinases in having a PH domain, which mediates membrane targeting by binding PIP3. This exposes the kinase domain to membrane-associated Src (or a Src family kinase), which phosphorylates a regulatory tyrosine in the catalytic site (Tyr-566 in Bmx) to activate the kinase (9). Tec kinases also contain SH2 and SH3 domains, and the latter SH3 domain may mediate an intramolecular inhibitory interaction with the PH domain that is also regulated by tyrosine phosphorylation (10–13). Bmx was originally cloned as a novel tyrosine kinase expressed in myeloid cells, but in contrast to other Tec kinases with limited tissue distribution, it is expressed in many cell types, including arterial endothelium and epithelial cells (14–17). Bmx can be activated by diverse stimuli, including cytokines and growth factors, and it has been implicated in cellular functions, including differentiation, motility, and apoptosis (14, 18–25).

Bmx in myeloid cells can be activated by multiple cytokines, and this activation is PI3-kinase-dependent (26). In contrast, Bmx in endothelial cells is activated by tumor necrosis factor (TNF) through an interaction with the type 2 TNF receptor (TNFR2), with subsequent Bmx-mediated stimulation of the VEGF receptor 2 (VEGFR2, KDR) and PI3-kinase by unclear mechanisms (22, 27). Significantly, Bmx-deficient mice have a defect in ischemia-mediated angiogenesis that correlates with decreased TNFR2 and VEGFR2 signaling in endothelium and bone marrow cells, indicating that Bmx functions physiologically through these receptors in endothelial cells (28). In epithelial...
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Bmx colocalizes with focal adhesion kinase (FAK) in response to integrin signaling, and FAK-mediated phosphorylation of tyrosine 40 in the Bmx PH domain may prime Bmx for subsequent phosphorylation by Src (29). Bmx may then stimulate migration by phosphorylation of p130Cas (30) and may interact with or activate additional proteins that regulate motility (RhoA, Rap1, and PAK1) (20, 31–33) or vesicular trafficking (caveolin and RUFY1) (34, 35). Bmx has also been reported to phosphorylate multiple Stats (Stat1, -3, and -5), and Bmx was necessary for Src-mediated activation of Stat3 and transformation in epithelial cells (23, 24, 36).

Bmx was identified in PCa through screens to detect tyrosine kinases expressed in PCa cell lines and clinical samples, and a recent immunohistochemical study indicates that its expression is increased in PCa relative to normal prostate epithelium (16, 17, 37). Bmx in PCa cells can be activated by IL-6 or by a constitutively active p110 subunit of PI 3-kinase, and Bmx activation by IL-6 is PI 3-kinase-dependent (19, 38). Neuropeptides can also activate Bmx in PCa cells, and this activation is dependent on both FAK and Src but is independent of PI 3-kinase (21). Overexpression of Bmx can protect PCa cells from radiation or drug-induced apoptosis, and this effect may be mediated through an interaction with the serine/threonine kinase Pim1 (38–40). Significantly, Src kinase SH3 domains can also mediate an interaction with p53, and Bmx can suppress p53 transcriptional activity, indicating that Bmx may prevent apoptosis through p53 (41). Finally, a recent study showed that transgenic overexpression of Bmx in mouse prostate epithelium caused increased proliferation and the development of lesions resembling human PIN, further indicating that Bmx may be a critical target of PI 3-kinase pathway activation in PCa (37).

In this study we first confirmed that Bmx was activated downstream of the constitutively active PI 3-kinase in PTEN-deficient PCa cells, and that down-regulation of Bmx could markedly suppress PCa cell growth. We then used Bmx immunoprecipitation and Tyr(P) immunoblotting to identify candidate Bmx upstream activators or downstream substrates in PCa cells, which revealed an interaction between Bmx and ErbB3. Significantly, heregulin-β1 treatment increased the Bmx interaction with ErbB3 and enhanced Bmx tyrosine phosphorylation and kinase activity. Moreover, EGF treatment similarly enhanced Bmx activation and induced an interaction between Bmx and EGFR. Bmx activation by heregulin-β1 was dependent on both Src and PI 3-kinase, whereas Bmx activation by EGF was Src-dependent but PI 3-kinase-independent. These studies show that Bmx is a downstream target of the activated PI 3-kinase pathway in PTEN-deficient PCa cells, and they provide the first direct link between activation of Bmx and receptor tyrosine kinases. Moreover, they indicate that Bmx should be a valuable therapeutic target in PCa and other cancers in which PI 3-kinase and EGF family receptor pathways are activated.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Mouse anti-Bmx antibody was from BD Biosciences. Mouse anti-phosphotyrosine antibody (4G10) and 4G10-conjugated agarose beads were from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse anti-ErbB3 (2B5) antibody was from Lab Vision Corp. (Fremont, CA). Rabbit polyclonal antibodies raised against EGF receptor (EGFR), phospho-EGFR (Tyr-1068), ErbB2/Her2, Akt, phospho-Akt (Ser-473), Stat3, and phospho-Stat3 (Tyr-705) were from Cell Signaling (Danvers, MA). Mouse anti-FLAG antibody (M2)-conjugated agarose beads, 3xFLAG peptide, IL-6, and PI 3-kinase inhibitor LY294002 were from Sigma. Mouse anti-β-tubulin antibody was from Chemicon (Temecula, CA). Horseradish peroxidase-conjugated mouse IgG and rabbit IgG were from Promega (Madison, WI). Recombinant human heregulin-β1 and EGF were from R & D Systems (Minneapolis, MN). Src inhibitor PP2 was from Calbiochem. Protease inhibitor mixture tablets (EDTA-free) were from Roche Diagnostics. Bmx siRNA and control siRNAs were from Dharmacon RNA Technologies (Lafayette, CO). Bmx cDNA with 3xFLAG sequence at the 5′ end was inserted into the pcDNA3 vector (Invitrogen) between NheI and NotI sites.

Cell Culture and Transfection—The PTEN-deficient LNCaP and PC3 human PCa cell lines, and the CWR22Rv1 PCa line with intact PTEN, were from the ATCC and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). For transfectants, 10 μg of 3xFLAG-Bmx/pcDNA3 DNA, HA-Bmx/pcDNA3, or PTEN-pcDNA DNA were transfected into LNCaP or PC3 cells in 10-cm plates at 50% confluence using Lipofectamine, with empty vector for controls. For stable transfectants, the media were replaced the next day, and cells were selected with G418 (900 μg/ml). Resistant cells were maintained in medium containing 500 μg/ml G418, and the expression of 3xFLAG-Bmx or HA-Bmx was analyzed by immunoblotting. Bmx knockdowns, siRNAs were transfected into LNCaP cells at 40 nM, and media were replaced 24 h later. Cells were either maintained in RPMI 1640 medium supplemented with 10% FBS for 4 days or in serum-containing medium for 2 days followed by serum starvation for 2 days. Cells were then trypsinized and counted.

Immunoprecipitation and Immunoblotting—Cells were treated with growth factors, inhibitors, or vehicle (0.1% Me2SO) as indicated. They were then washed twice with ice-cold Tris-buffered saline (TBS) and lysed with 1 ml of ice-cold RIPA buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 1 mM pyrophosphate, 100 mM sodium fluoride, 1 mM NaVO4, and a mixture of protease inhibitors). Cell lysates were sonicated for 10 s and centrifuged at 13,000 rpm at 4°C for 15 min to remove cell debris. For anti-Tyr(P) immunoprecipitations, supernatants were transferred to new microcentrifuge tubes, and equal amounts of proteins (1–5 mg) from each sample were mixed with 20–50 μl of 4G10-conjugated agarose beads and incubated at 4°C overnight with continuous agitation. The mixtures were then transferred to MicroSpin columns (GE Healthcare). The beads were washed with 600 μl of RIPA buffer six times followed by washing with 600 μl of TBS twice. Subsequently, the columns were placed into microcentrifuge tubes and spun at 1200 rpm for 30 s to remove remnants of TBS. The bottoms of the columns were plugged, and 10 μl of 2X Laemmli sample buffer without β-mercaptoethanol were added to each column followed by placing the capped columns into microcentrifuge tubes. After
incubating at 65 °C for 15 min (to elute bound proteins), the columns were unplugged, transferred into new microcentrifuge tubes, and spun at 2000 rpm for 30 s to collect samples. 1 μl of β-mercaptoethanol was added to each sample, and the samples were boiled for 5 min. Samples were then resolved by 4–12% NuPAGE gel (Invitrogen) followed by membrane transfer. The membranes were blocked with 5% milk in TBS containing 0.1% Tween 20 (TBS/T) at room temperature for 2 h and incubated with primary antibodies overnight at 4 °C. After washing with TBS/T, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h, washed, and developed with enhanced chemiluminescence (ECL) reagents (PerkinElmer Life Sciences).

For Bmx immunoprecipitations by M2-conjugated agarose beads, cells were lysed with TBS containing 1% Triton X-100, 1 mM Na3VO4, and a mixture of protease inhibitors. Equal amounts of proteins (1–5 mg) from each sample were then mixed with 20–50 μl of M2-conjugated agarose beads and incubated at 4 °C for 2 h with continuous agitation. The beads were then transferred to MicroSpin columns, washed, and incubated with 10 μl of 3xFLAG peptide (100 μg/ml) at 4 °C overnight with continuous agitation to elute 3xFLAG-Bmx and associated proteins. The columns were spun at 2000 rpm for 30 s to collect samples. 3 μl of 6X Laemmli sample buffer and 1 μl of β-mercaptoethanol were added to each sample, and the samples were boiled for 5 min. Electrophoresis and immunoblotting were then performed as described above.

For immunoprecipitation of endogenous Bmx, cells were lysed with TBS containing 1% Triton X-100, 1 mM Na3VO4, and a mixture of protease inhibitors. 5 mg of proteins from each sample were mixed with 5 μg of mouse anti-Bmx antibody (BD Biosciences) and 5 μg of rabbit anti-Bmx antibody (Cell Signaling) and then incubated overnight at 4 °C with continuous agitation. Equal amounts of mouse IgG and rabbit IgG were used as negative controls. 20 μl of protein A/G beads (Pierce) were then added to each sample and incubated at 4 °C for 2 h with continuous agitation. Elution of the immunocomplexes and electrophoresis and immunoblotting were performed as described above.

**In Vitro Kinase Assay**—3xFLAG-Bmx was immunoprecipitated from LNCaP cells, treated with or without growth factors or LY294002, and eluted with 3xFLAG peptide. Eluted Bmx was then mixed with kinase buffer (final 20 mM HEPES, pH 7.5, 10 mM MgCl2, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 20 μM ATP, 5 mM Na3VO4) and 1 μCi of [γ-32P]ATP (PerkinElmer Life Sciences). The kinase assay was performed at 30 °C for 30 min and stopped with 10 mM EDTA and Laemmli sample buffer. The samples were resolved by 4–12% NuPAGE gel, and Bmx autophosphorylation was visualized by autoradiography.

**RESULTS**

**Bmx Is Constitutively Activated Downstream of PI 3-Kinase and Src in PTEN-deficient PCA Cells**—PTEN-deficient LNCaP PCa cells have high levels of constitutive PI 3-kinase pathway activation even when cultured in serum-free medium, and inhibition of PI 3-kinase with LY294002 or wortmannin markedly suppresses LNCaP cell growth. To determine whether Bmx was constitutively activated by endogenous PI 3-kinase in PTEN-deficient cells, we generated LNCaP cells that were stably transfected with an epitope-tagged (3xFLAG) Bmx, which could be efficiently immunoprecipitated using anti-FLAG antibodies. As noted above, LNCaP cells grown in serum-free medium maintained high levels of PI 3-kinase pathway activation, as assessed by immunoblotting for pAkt (Fig. 1A). Immunoprecipitation of Bmx from these cells grown in serum-free medium followed by anti-Tyr(P) immunoblotting indicated that Bmx was partially activated, and it could be further activated when the cells were grown in serum-containing medium (10% FBS) (Fig. 1A, lanes 1 and 3). Consistent with previous data, IL-6 treatment rapidly increased Bmx phosphorylation and Stat3 activation (Fig. 1A, lane 2). Significantly, treatment with a PI 3-kinase inhibitor (LY294002) abrogated Akt activation and decreased the levels of Bmx tyrosine phosphorylation, indicating that PI 3-kinase contributes to Bmx activation under these conditions (Fig. 1A, lanes 4 versus 1). The basal level of Bmx tyrosine phosphorylation was also reduced by an Src inhibitor (Fig. 1A, PP2, lane 5), and the combination of LY294002 and PP2 resulted in nearly complete loss of basal Bmx phosphorylation (lane 6).

When cells were grown in serum-containing medium (10% FBS), we also found that Bmx tyrosine phosphorylation could be markedly decreased by treatment with LY294002 and PP2 (Fig. 1B). Similar results were obtained in other independent stable LNCaP clones expressing FLAG-Bmx or expressing an HA-tagged Bmx (Fig. 1C and data not shown). We also examined another PTEN-deficient PCa cell line, PC3, which was stably transfected with the 3xFLAG-Bmx. In these cells PP2 alone was more effective than LY294002, but the combination completely blocked Bmx phosphorylation (Fig. 1D).

We next used anti-Tyr(P) immunoprecipitation followed by Bmx immunoblotting to assess the activation of endogenous Bmx in LNCaP cells. There was no detectable activation of endogenous Bmx (above the basal level) by IL-6 in serum-starved cells, although the IL-6 still markedly activated Stat3 (Fig. 1E, lane 2). Similarly to the transfected Bmx, tyrosine phosphorylation of endogenous Bmx was increased by growth in serum-containing medium. Importantly, inhibition of PI 3-kinase or Src with LY294002 or PP2, respectively, markedly decreased the phosphorylation of endogenous Bmx in serum-containing medium. Moreover, the combination of LY294002 and PP2 decreased Bmx phosphorylation to below its basal level in serum-free medium.

In contrast to these results in PTEN-deficient LNCaP cells, tyrosine phosphorylation of Bmx could not be detected under the same conditions in a PCa cell line with intact PTEN (CWR22Rv1), although endogenous Bmx could be readily detected in whole cell lysates from these cells (Fig. 1F). Finally, transfection of wild-type PTEN into the 3xFLAG-Bmx expressing LNCaP cells markedly reduced both PI 3-kinase pathway activation, as assessed by pAkt immunoblotting, and tyrosine phosphorylation of Bmx (Fig. 1G). Taken together, these results indicated that Bmx was constitutively active in LNCaP PCa cells grown in serum-free medium, that it was further activated in medium with 10% FBS, and that the major pathway mediating Bmx activation was PI 3-kinase-
Identification of Bmx-associated Tyrosine-phosphorylated Proteins—In addition to membrane recruitment mediated by the Bmx PH domain, the SH2 domain presumably mediates Bmx binding to tyrosine-phosphorylated proteins that may be upstream activators or downstream targets of Bmx. To identify candidate proteins interacting with the Bmx SH2 domain, we used anti-FLAG antibody-conjugated beads to immunoprecipitate 3xFLAG epitope-tagged Bmx from stably transfected LNCaP cells. The beads were eluted sequentially with 3xFLAG peptide and then SDS, as a variable amount of the 3xFLAG Bmx remains associated with the beads after peptide elution. The eluted proteins were then immunoblotted with anti-Tyr(P). As a further control for proteins binding nonspecifically to the beads, we also carried out the anti-FLAG immunoprecipitations on LNCaP cells stably transfected with an empty expression vector.

Fig. 3A is a Tyr(P) immunoblot of whole cell lysates from Bmx (B) and vector control (V) stably transfected LNCaP cells, before and after the anti-FLAG immunoprecipitation. A large number of tyrosine-phosphorylated proteins were observed in both cells, with several bands appearing to be increased in the Bmx-expressing cells. Strikingly, a single major tyrosine-phosphorylated protein of ~180 kDa was detected in the peptide eluted material from the 3xFLAG-Bmx expressing LNCaP cells versus the control cells (Fig. 3B). Further elution with SDS revealed additional specific bands at ~110 and ~80 kDa. Similar bands were obtained by peptide and/or SDS elution in independent experiments, with the upper band at ~180 kDa being most prominent (Fig. 3C). The tyrosine-phosphorylated band migrating at ~80 kDa was consistent with the epitope-tagged Bmx, which migrates at this position. It should again be noted that although the 3xFLAG peptide elution is very specific, it only removes a variable fraction of the 3xFLAG-Bmx, and the tyrosine-phosphorylated fraction may elute less readily.

We then carried out further immunoblotting of candidate proteins to identify the major tyrosine-phosphorylated protein(s) at ~180 kDa. Significantly, ErbB3 (~185 kDa) was found by immunoblotting to be highly enriched in the specific peptide and SDS-eluted material (Fig. 3D). ErbB3 can be phosphorylated by ErbB2 and EGFR in response to growth factor stimulation and can form heterodimers with these receptor tyrosine kinases. However, we did not detect a consistent interaction...
between Bmx and ErbB2 or EGFR in unstimulated cells (see Fig. 6). These results indicated that ErbB3 was associated with Bmx in LNCaP cells, although there may clearly be additional associated proteins at ~180 kDa or other molecular masses.

**Bmx Is Activated by Heregulin-β1**—The interaction between Bmx and ErbB3 suggested that ErbB2, the major kinase mediating ErbB3 phosphorylation, may be a direct or indirect upstream activator of Bmx. To test this hypothesis, we assessed tyrosine phosphorylation of the 3xFLAG-Bmx in serum-starved LNCaP cells in response to heregulin-β1, which stimulates ErbB2/ErbB3 dimerization and ErbB2 kinase activity. As expected, heregulin-β1 treatment stimulated the tyrosine phosphorylation of ErbB2 and ErbB3, as assessed by anti-Tyr(P) immunoprecipitation and immunoblotting, with the increased ErbB3 tyrosine phosphorylation persisting throughout the time course (Fig. 4A). Significantly, this correlated with a rapid increase in Bmx tyrosine phosphorylation, which peaked within 15 min and then declined to below base-line levels over 8 h. This decline was associated with a decrease in total Bmx, and declines in total and phosphorylated ErbB2, but not ErbB3.

To confirm that the anti-Tyr(P) immunoprecipitation of Bmx was because of tyrosine phosphorylation of Bmx, versus Bmx association with other tyrosine-phosphorylated proteins, we directly immunoprecipitated Bmx with anti-FLAG beads. Immunoblotting with Tyr(P) then confirmed that Bmx tyrosine phosphorylation was rapidly and strongly increased in response to heregulin-β1 (Fig. 4B). Interestingly, we also detected an increased association between Bmx and a tyrosine-phosphorylated ~185-kDa protein in response to heregulin-β1, which was consistent with ErbB3 (see Fig. 6).

Finally, we determined whether endogenous Bmx was similarly activated by heregulin-β1. Nontransfected LNCaP cells were serum-starved and then stimulated with heregulin-β1 for 15 min to 8 h, and lysates were immunoprecipitated with anti-Tyr(P). Immunoblotting confirmed the expected activation of ErbB2, with a marked increase in ErbB3 phosphorylation (Fig. 4C). Interestingly, phosphorylation of ErbB2 was more persistent, whereas ErbB3 was more transient in these cells versus the Bmx overexpressing LNCaP cells, although we do not know if this is a direct effect of increased Bmx. In any case, consistent with the above data, there was also a marked increase in the phosphorylation of endogenous Bmx in response to heregulin-β1. Taken together, these data indicated that ErbB2/ErbB3 may mediate the recruitment and activation of Bmx.

**Bmx Is Activated by EGF**—Although we did not detect an interaction between Bmx and EGFR in LNCaP cells in 10% serum, a previous study in breast cancer cells found that transfected Bmx could be activated by EGF treatment (42). Therefore, similar experiments were carried out to examine Bmx activation in response to EGF. Treatment of LNCaP cells with EGF resulted in a rapid (within 5 min) increase in tyrosine phosphorylation of EGFR and subsequent receptor down-regulation, with a similar increase in ErbB2 phosphorylation that presumably reflects EGFR/ErbB2 heterodimerization (Fig. 5A). Phosphorylation of ErbB3 was only transiently increased, versus the persistent increase observed above with heregulin-β1. Significantly, Bmx tyrosine phosphorylation was also increased within 5 min by EGF, which was confirmed by first immunoprecipitating Bmx with anti-FLAG and then immunoblotting for Tyr(P) (Fig. 5B). In contrast to stimulation with heregulin-β1, Bmx phosphorylation in response to EGF was more transient and did not result in a decrease in total Bmx. A tyrosine-phosphorylated band consistent with EGFR or ErbB3 was also associated with Bmx after EGF stimulation (see Fig. 6).

We next examined tyrosine phosphorylation of endogenous Bmx in response to EGF stimulation. Nontransfected LNCaP cells were serum-starved and then stimulated with EGF for 5–120 min, and lysates were then immunoprecipitated with anti-Tyr(P) and immunoblotted. EGF again stimulated marked increases in the phosphorylation of EGFR, ErbB2, and ErbB3 in these nontransfected LNCaP cells (Fig. 5C). Significantly, EGF...
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also stimulated a rapid increase in the tyrosine phosphorylation of endogenous Bmx. Taken together, these results show that EGFR can mediate Bmx recruitment and activation, although the activation of both EGFR and ErbB2 in response to EGF indicates that this Bmx activation might be ErbB2-mediated.

Finally, we carried out in vitro kinase assays to confirm that the increased tyrosine phosphorylation of Bmx correlated with increased kinase activity. Serum-starved LNCaP cells were stimulated with EGF or heregulin-β1 and then immunoprecipitated with anti-FLAG. Bmx was peptide eluted, and autophosphorylation activity was assessed by incubation in kinase buffer with \([\text{32P}]\text{ATP}\). Consistent with the Tyr(P) immunoblotting, basal kinase activity was precipitated by anti-FLAG from the 3xFLAG-Bmx expressing LNCaP cells, and this could be rapidly increased by EGF or heregulin-β1 (Fig. 5D).

**FIGURE 5. Bmx is activated by heregulin-β1**. A and B, Bmx activation in LNCaP-FLAG-Bmx cells. Cells were serum-starved for 2 days and then stimulated with heregulin-β1 (40 ng/ml) for 0.25, 0.5, 1, 2, or 4 h. A, cells were lysed with RIPA buffer containing a mixture of protease inhibitors and 1 mM Na3VO4. 5 mg of cell lysates were incubated with 50 μl of 4G10-conjugated agarose beads overnight at 4 °C, and SDS eluted material was immunoprecipitated with Bmx, ErbB2, or ErbB3 antibodies. Total protein levels of Bmx, ErbB2, and ErbB3 were also measured by immunoblotting. B, reciprocal immunoprecipitation of Bmx. Cell lysates were immunoprecipitated (IP) with anti-FLAG, peptide-eluted, and immunoblotted with 4G10 antibody. Molecular standards are indicated at the left margin. C, endogenous Bmx activation in untransfected LNCaP cells. Growth factor stimulation, immunoprecipitations, and subsequent immunoblotting were performed the same as described in A. Bar graphs show the ratios of anti-Tyr(P) or anti-Bmx versus anti-tubulin band intensities, and data are representative of at least three experiments.

3xFLAG-tagged Bmx, EGF increased Bmx tyrosine phosphorylation and stimulated an interaction between the FLAG-Bmx and EGFR that was not detectable in the unstimulated cells and was substantial relative to the decreased levels of EGFR after EGF stimulation. In contrast, EGFR did not stimulate interactions with ErbB2 or ErbB3, and no interactions in the control Ab precipitates (Fig. 6B and data not shown).

Serum-starved LNCaP cells were analyzed similarly after stimulation with EGF. As shown in Fig. 6C using

**FIGURE 4. Bmx is activated by heregulin-β1**. A and B, Bmx activation in LNCaP-FLAG-Bmx cells. Cells were serum-starved for 2 days and then stimulated with heregulin-β1 (40 ng/ml) for 0.25, 0.5, 1, 2, or 4 h. A, cells were lysed with RIPA buffer containing a mixture of protease inhibitors and 1 mM Na3VO4. 5 mg of cell lysates were incubated with 50 μl of 4G10-conjugated agarose beads overnight at 4 °C, and SDS eluted material was immunoprecipitated with Bmx, ErbB2, or ErbB3 antibodies. Total protein levels of Bmx, ErbB2, and ErbB3 were also measured by immunoblotting. B, reciprocal immunoprecipitation of Bmx. Cell lysates were immunoprecipitated (IP) with anti-FLAG, peptide-eluted, and immunoblotted with 4G10 antibody. Molecular standards are indicated at the left margin. C, endogenous Bmx activation in untransfected LNCaP cells. Growth factor stimulation, immunoprecipitations, and subsequent immunoblotting were performed the same as described in A. Bar graphs show the ratios of anti-Tyr(P) or anti-Bmx versus anti-tubulin band intensities, and data are representative of at least three experiments.
FIGURE 6. Bmx association with ErbB3 and EGFR is enhanced by growth factor stimulation. LNCaP-FLAG-Bmx cells (A and C) or nontransfected LNCaP (B and D) cells were serum-starved for 2 days and then stimulated with heregulin-1 (40 ng/ml; 0.25, 0.5, or 1 h; A and B) or EGF (20 ng/ml; 5, 15, 30 min; C and D). A and C, 3xFLAG Bmx was immunoprecipitated (IP) from 3 mg of cell lysates using 30 μl of M2-conjugated agarose beads. Peptide eluted protein migrating at ~80 kDa was then immunoblotted with 4G10 (upper panels), and eluted proteins migrating between ~120–220 kDa were blotted for EGF, ErbB2, or ErbB3 as indicated. Total protein levels of Bmx, EGFR, ErbB2, ErbB3, and β-tubulin in the whole cell lysates (1% of input) were also analyzed by immunoblotting. B and D, endogenous Bmx was immunoprecipitated from LNCaP cell lysates using anti-Bmx or negative control antibodies and blotted for EGF, ErbB2, and ErbB3, as indicated (no bands were detected in the negative control antibody immunoprecipitates; data not shown). Total EGFR, ErbB2, ErbB3, and β-tubulin in 1% of the cell lysates used for immunoprecipitation are also shown. Data are representative of two (B and D) or three (A and C) experiments.

FIGURE 7. Bmx phosphorylation in response to heregulin-1 is Src- and PI 3-kinase-dependent. A, LNCaP-FLAG-Bmx cells were serum-starved for 2 days. Heregulin-1 (40 ng/ml) stimulation of the cells was performed 15 or 30 min before the end of a 2-h incubation with LY294002 (LY, 20 μM) or PP2 (10 μM). Bmx was immunoprecipitated (IP) from 1 mg of cell lysates by using 20 μl of M2-conjugated agarose beads. Peptide eluted Bmx was analyzed by immunoblotting with 4G10 antibody. B, nontransfected LNCaP cells were serum-starved for 2 days and treated with LY294002, PP2, or heregulin-1 as described in A. Tyrosine-phosphorylated proteins were immunoprecipitated from 5 mg of cell lysates by 30 μl of 4G10-conjugated agarose beads, and SDS eluted proteins were immunoblotted with Bmx antibody. Immunoblotting with pAkt was performed to assess the activity of PI 3-kinase. Bar graphs show the ratios of anti-Tyr(P) or anti-Bmx versus anti-tubulin band intensities, and data are representative of at least three experiments.

Bmx phosphorylation in response to heregulin-1 is Src- and PI 3-Kinase-dependent—Although these results showed that EGFR and ErbB2 could mediate Bmx recruitment, it was not clear whether they also mediated Bmx phosphorylation and activation independently of PI 3-kinase or Src. Therefore, we next determined whether LY294002 or PP2 could block Bmx phosphorylation in response to heregulin-1. Brief pretreatment of serum-starved 3xFLAGBmx-LNCaP cells with LY294002 (0.5–2 h) markedly decreased PI 3-kinase activity, as assessed by Akt activation, although heregulin-1 still caused a slight increase in Akt activation (Fig. 7A). LY294002 treatment for 2 h modestly decreased basal activation of Bmx, as assessed by anti-FLAG immunoprecipitation and anti-Tyr(P) immunoblotting, and more substantially decreased Bmx phosphorylation in response to heregulin-1 (Fig. 7A). Significantly, LY294002 also abrogated the phosphorylation of endogenous Bmx in response to heregulin-1, as assessed by anti-Tyr(P) immunoprecipitation and Bmx immunoblotting (Fig. 7B). Interestingly, although endogenous Bmx phosphorylation was blocked, LY294002 again did not completely block heregulin-1-mediated activation of Akt.

Treatment with PP2 more markedly suppressed basal phosphorylation of 3xFLAG and endogenous Bmx and strongly inhibited Bmx phosphorylation in response to heregulin-1 (Fig. 7, A and B). In contrast, heregulin-1-mediated activation of Akt was not blocked by PP2 (Fig. 7, A and B). Taken together, these data indicate that Bmx activation by heregulin-1 is dependent on both PI 3-kinase and Src (or an Src family kinase).

Bmx Phosphorylation in Response to EGF Is Src-dependent and PI 3-Kinase-independent—Similarly to its effect on heregulin-1 stimulation, PP2 abrogated the phosphorylation of 3xFLAG and endogenous Bmx in response to EGF (Fig. 8, A and B). In contrast, LY294002 did not block EGF-stimulated phosphorylation of transfected nor endogenous Bmx (Fig. 8, A and B). Significantly, this did not appear to be because of residual PI 3-kinase activation, as basal and EGF-stimulated Akt phosphorylation were almost completely suppressed by the LY294002 treatment.

Finally, to further assess whether Bmx activation by EGF was PI 3-kinase-independent, we carried out in vitro kinase assays. Serum-starved 3xFLAGBmx-LNCaP cells were EGF-stimulated, and Bmx was isolated by anti-FLAG immunoprecipitation and 3xFLAG peptide elution. Bmx kinase activity, as assessed by autophosphorylation in in vitro kinase assays, was rapidly stimulated by EGF (Fig. 8C). Treatment with LY294002 decreased basal Bmx kinase activity and completely suppressed Akt phosphorylation, but it did not inhibit Bmx activation in response to EGF. These data are consistent with the above Bmx tyrosine phosphorylation results and confirm that the rapid activation of Bmx in response to EGF is PI 3-kinase-independent.
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FIGURE 8. Bmx phosphorylation in response to EGF is Src-dependent and PI 3-kinase-independent. A, LNCaP-FLAG-Bmx cells were serum-starved for 2 days. EGF (20 ng/ml) stimulation of the cells was performed 5 or 30 min before the end of a 2-h incubation with LY294002 (20 μM) or PP2 (10 μM). Bmx was immunoprecipitated (IP) from 1 mg of cell lysates by using 20 μl of M2-conjugated agarose beads, eluted from the M2 beads by FLAG peptide, and analyzed by immunoblotting with 4G10 antibody. B, nontransformed LNCaP cells were serum-starved for 2 days and treated with LY294002, PP2, or EGF the same as described in A. Cell lysates (5 mg) were immunoprecipitated with 30 μl of 4G10-conjugated agarose beads, SDS eluted, and immunoblotted with Bmx antibody. Bar graphs show the ratios of anti-Tyr(P) or anti-Bmx versus anti-tubulin band intensities, and data are representative of at least three experiments. C, in vitro kinase assay. LNCaP-FLAG-Bmx cells were treated and immunoprecipitated as described in A. Peptide eluted Bmx was mixed with kinase buffer, and the kinase assay was performed at 30 °C for 30 min. The samples were resolved by 4–12% NuPAGE gel, and Bmx autophosphorylation was visualized by autoradiography. Bar graph shows the relative intensities of the labeled bands. Whole cell lysates were immunoblotted with Bmx and anti-p-Akt antibody to confirm inhibition of PI 3-kinase.

DISCUSSION

PTEN loss and PI 3-kinase activation make a major contribution to PCa, and pathways activated downstream of PI 3-kinase are candidate therapeutic targets. This study shows that Bmx is activated downstream of the constitutively active PI 3-kinase pathway in PTEN-deficient LNCaP and PC3 PCa cells, and that Bmx down-regulation by RNA interference markedly inhibits cell growth, indicating that Bmx is a critical downstream effector of PI 3-kinase in these PCa cells. Moreover, Bmx was further rapidly stimulated in response to activation of ErbB2/ErbB3 and EGFR in PCa cells. Treatment of LNCaP cells with an ErbB2/ErbB3 ligand, heregulin-β1, enhanced an interaction between Bmx and ErbB3 and stimulated the activation of Bmx. EGF treatment similarly stimulated an interaction between EGFR and Bmx and rapid Bmx activation. Although heregulin-β1 stimulated a Bmx-ErbB3 interaction, Bmx activation by heregulin-β1 was still PI 3-kinase-dependent, as it was blocked by LY294002. In contrast, Bmx activation by EGF was PI 3-kinase-independent. Finally, Bmx activation in response to both heregulin-β1 and EGF was blocked by inhibition of Src, indicating that Src mediates Bmx phosphorylation subsequent to its membrane recruitment by PI 3-kinase, ErbB3, or EGFR. Taken together, these results show that Bmx is a critical downstream target of the constitutively active PI 3-kinase in PTEN-deficient PCa cells and demonstrate the first direct link between activation of Bmx and receptor tyrosine kinases. Moreover, they indicate that Bmx may be a valuable therapeutic target in PCa and other epithelial malignancies in which PI 3-kinase or EGFR receptor family pathways are activated.

The activation of Bmx and other Tec kinases is dependent on phosphorylation of a regulatory tyrosine in the catalytic site, which is mediated by Src or Src family kinases (9). Moreover, activation of Bmx and other Tec kinases by many stimuli is PI 3-kinase-dependent, although previous studies indicate that this activation in response to some stimuli may be independent of PI 3-kinase (21, 29, 43). In contrast to Src family kinases that are inhibited by an interaction between their SH2 domain and a C-terminal phosphotyrosine, Tec kinases may be autoinhibited by an SH3 domain-mediated intramolecular interaction (10–13). This inhibitory interaction in Bmx may be disrupted by PIP3 binding to the PH domain or by FAK binding to the PH domain and subsequent phosphorylation of Tyr-40 in the PH domain (29). Therefore, the PI 3-kinase dependence of herregulin-β1-mediated Bmx activation may reflect a requirement for PIP3 binding to expose the Bmx kinase domain for phosphorylation by Src. Alternatively, PIP3 may be required to stabilize the interaction between Bmx and ErbB3 or to enhance Bmx targeting to an Src-enriched membrane domain.

A final alternative is that Bmx is activated in response to herregulin-β1 independently of its recruitment by ErbB3, with ErbB3 serving as a scaffold or possibly a substrate for activated Bmx. In this regard, it may be significant that ErbB3 tyrosine phosphorylation in response to heregulin-β1 was persistent in the Bmx overexpressing LNCaP cells versus more transient in the control cells. In any case, as ErbB3 is a potent activator of PI 3-kinase, these data indicate that Bmx may be particularly responsive to PI 3-kinase activation through ErbB2/ErbB3 and
may function physiologically to integrate signaling through the PI 3-kinase and ErbB2/ErbB3 pathways. In contrast to Bmx activation by heresulin-β1, EGFR-mediated activation of Bmx was not dependent on PI 3-kinase. This may reflect Bmx targeting to a distinct membrane microdomain by EGFR or conformational changes mediated by EGFR (possibly because of phosphorylation at Tyr-40 or other sites) that expose the Bmx kinase domain for phosphorylation by Src, independently of PIP3 binding.

Previous studies have indicated that Bmx may be activated by receptor tyrosine kinases. Studies in endothelial cells showed that Bmx could be activated by VEGF or by transfected overexpressed Tie-2 and VEGF receptor 1 (14, 25). However, it is not known whether Bmx interacts directly with these receptors, and Bmx activation by the endogenous receptors has not been established. Another study in MDA-MB-468 breast cancer cells showed that EGFR could stimulate tyrosine phosphorylation of adenovirus transduced Bmx, which could be partially blocked by Src and PI 3-kinase inhibitors (42). This study also used a phospho-specific antibody to show EGFR stimulated phosphorylation of Tyr-40 in the Bmx PH domain, but it is not clear whether this is mediated by EGFR or another kinase, including FAK. Interestingly, EGFR stimulates Stat1 activation and apoptosis in these MDA-MB-468 cells, and both could be blocked by a kinase domain-deleted Bmx (42). Further studies are needed to determine whether these observations in MDA-MB-468 cells reflect a direct interaction between EGFR and Bmx and to determine the basis for the apoptotic response.

Bmx activation in PCa cells can also be stimulated by neurotrophines (bombesin and neurotensin) and IL-6, and Bmx is required for neuroendocrine differentiation induced by these agents in LNCaP cells (19, 21). Neuropeptide activation of Bmx is mediated by FAK and Src and is PI 3-kinase-independent. In contrast, IL-6-mediated Bmx activation is PI 3-kinase-dependent. We observed IL-6 activation of transfected Bmx, but not endogenous Bmx, despite robust Stat3 activation in response to IL-6. The basis for this difference is not clear. A previous study also indicated that Bmx is a mediator of Src-induced Stat3 activation, although these studies were done in rat hepatoma cells (36). In any case, given the marked effects of Bmx down-regulation, although these studies were done in rat hepatoma cells also indicated that Bmx is a mediator of Src-induced Stat3 activation and a possible Bmx substrate activated (36). In any case, given the marked effects of Bmx down-regulation, although these studies were done in rat hepatoma cells also indicated that Bmx is a mediator of Src-induced Stat3 activation and a possible Bmx substrate activated (36).

Finally, these studies indicate that Bmx may be activated and a therapeutic target in other epithelial cancers with increased ErbB2 or EGFR receptor activity.

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