PP2A-B55β Antagonizes Cyclin E1 Proteolysis and Promotes Its Dysregulation in Cancer

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Abstract

Cyclin E1 regulates the initiation of S-phase in cellular division. However, in many cancers, cyclin E1 is aberrantly overexpressed and this molecular phenotype correlates with increased tumor aggressiveness and poor patient survival. The molecular cause(s) of cyclin E1 abnormalities in cancers is poorly understood. Here, we show that cyclin E1 overexpression in cancer is promoted by dysregulation of the protein phosphatase PP2A-B55β. PP2A-B55β targets the N- and C-terminal phosphodegrons of cyclin E1 for dephosphorylation, thus protecting it from degradation mediated by the SCFFbw7 ubiquitin ligase. Augmented B55β expression stabilizes cyclin E1 and promotes its overexpression in cancer-derived cell lines and breast tumors. Conversely, B55β ablation enforces the degradation of cyclin E1 and inhibits cancer cell proliferation in vitro and tumor formation in vivo. Therefore, PP2A-B55β promotes cyclin E1 overexpression by antagonizing its degradation and its inhibition could represent a therapeutic mechanism for abrogating cyclin E1 function in cancers. Cancer Res; 74(7); 2006–14. ©2014 AACR.

Introduction

Cyclin E1, an activator of cyclin-dependent kinase 2 (CdK2), is an important regulator of the G1–S-phase transition of the cell division cycle. Cyclin E1 initiates an S-phase program by promoting histone biosynthesis, centrosome duplication, and licensing of DNA origins of replication, among other functions (1). Cyclin E1 protein level is tightly regulated during cellular division through coordinated transcriptional and proteolytic controls, which ensure its expression is restricted to the G1–S-phase transition. CCNE1, which encodes cyclin E1, is expressed in late G1 phase stimulated by E2F and c-Myc activities through controls, which ensure its expression is restricted to the G1–S-phase transition. Therefore, PP2A-B55β targets the N- and C-terminal phosphodegrons of cyclin E1 for dephosphorylation, thus protecting it from degradation mediated by the SCFFbw7 ubiquitin ligase. Augmented B55β expression stabilizes cyclin E1 and promotes its overexpression in cancer-derived cell lines and breast tumors. Conversely, B55β ablation enforces the degradation of cyclin E1 and inhibits cancer cell proliferation in vitro and tumor formation in vivo. Therefore, PP2A-B55β promotes cyclin E1 overexpression by antagonizing its degradation and its inhibition could represent a therapeutic mechanism for abrogating cyclin E1 function in cancers. Cancer Res; 74(7); 2006–14. ©2014 AACR.

A wealth of clinical studies have shown that cyclin E1 is aberrantly overexpressed in many human cancers and this molecular phenotype often correlates with increased tumor aggressiveness and poor patient survival. For example, approximately 30% of breast cancers overexpress cyclin E1 and this molecular abnormality has been shown to be a stronger prognostic marker of poor patient outcome than important clinicopathologic factors such as tumor grade/stage, hormone receptor status, or lymph node metastasis (5). Cyclin E1 overexpression is also a major molecular determinant of basal-like triple negative breast cancers [TNBC; estrogen receptor (ER−), progesterone receptor (PR−), and Her2−], a highly aggressive breast cancer subtype that is common in BRCA1 mutation carriers, and is an independent predictor of poor patient outcome (6). Cyclin E1 overexpression has also been associated with increased resistance to the anti-Her2 therapy trastuzumab in breast cancers (7).

At the molecular level, cyclin E1 overexpression alters gene expression programs that regulate cellular division, differentiation, survival, and senescence (1). It has also been shown to promote genetic instability by causing defects in chromosome segregation (8–10), centrosome hyperamplification (11), interference with the prereplication complex assembly (12), and formation of unstable DNA replication intermediates (13, 14).

Despite the importance of cyclin E1 in cancer, the molecular mechanisms that promote its dysregulation are poorly understood. In breast cancers, genetic amplifications of CCNE1 occur in only approximately 5% of cases (15). Furthermore, mutations of FBXW7, which encodes the substrate recognition component of SCFFbw7, are exceedingly rare (<1%; ref. 16). Therefore, other molecular mechanisms must be responsible for cyclin E1 overexpression in these cancers, which allow for its hyperaccumulation in the presence of functional degradation machinery.
Materials and Methods

Patient specimens and cell lines
Breast cancer specimens were obtained from patients treated at the Department of Obstetrics and Gynecology of the Innsbruck Medical University in Austria using Institutional Review Board–approved procedures. Clinicopathologic information for tumor specimens is provided in Supplementary Table S1. Cell lines were purchased from the American Tissue Culture Collection and grown in the recommended culture mediums. Detailed information about tumor analysis, cell line treatments, and plasmid and siRNAs transfections is provided in Supplementary Material and Methods.

Ubiquitylation assays
In vitro ubiquitylation assays were performed by cotransfecting HEK293T cells with the indicated expression plasmids and siRNAs and 48 hours later treating cells with 10 μmol/L MG132 for 4 hours before harvesting. Cells were lysed using denaturing conditions (1% SDS) as described (17). The Thr77Ala/Thr395Ala mutant of cyclin E1 was created using the Quickchange Site-Directed Mutagenesis Kit (Agilent). In vitro ubiquitylation reactions were performed as described previously (18). Human recombinant SCFFbxw7 was generated by multi-infecting Sf9 insect cells with the relevant expressing baculoviruses. Recombinant cyclin E1-Cdk2 was purchased from EMD Millipore.

Cell fractionation
Cells were suspended in buffer containing 10 mmol/L Tris (pH 7.4), 5 mmol/L MgCl2, 10 mmol/L KCl, 300 mmol/L sucrose, 0.1 mmol/L EDTA, 0.5 mmol/L dithiothreitol, with added phosphatase and protease inhibitors and incubated on ice for 10 minutes. NP-40 was then added to a final concentration of 0.125%, the cells vortexed for 10 seconds, and the cytoplasmic fraction clarified by centrifugation at 1,000 rpm in a microfuge. The nuclear pellet was then washed 4× in buffer containing 10 mmol/L Hepes (pH 7.4), 50 mmol/L NaCl, 25% glycerol, and 0.1 mmol/L EDTA, with the last wash spun at 6,000 rpm in a microfuge. The nuclear fraction was then isolated by incubation in buffer containing 20 mmol/L Tris (pH 7.4), 5 mmol/L MgCl2, 300 mmol/L KCl, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 25% glycerol, with phosphatase and protease inhibitors for 15 minutes, and the lysate clarified by centrifugation at 13,000 rpm for 15 minutes.

Phosphatase and kinase assays
For phosphatase assays, human recombinant cyclin E1-Cdk2 was incubated with PP2A core enzyme (EMD Millipore) in phosphatase reaction buffer (12.5 mmol/L Tris (pH 7.0), 25 μmol/L CaCl2) for 30 minutes at 30°C. Okadaic acid (EMD Millipore) and λPPase (New England Biolabs) were added to the indicated reactions. For PP2A-B55δ phosphatase assays, PP2A-B55δ complexes were assembled in HEK293T cells, immunopurified, and reactions performed as described above using recombinant cyclin E1-Cdk2 as substrate. Anti-Flag antibodies were purchased from Sigma-Aldrich. Kinase assays were performed by incubating human recombinant pRB with cyclin E1 immunoprecipitated from cell extracts (250 μg) with antibody HE172. Reactions were performed at 30°C for 30 minutes in reaction buffer containing 20 mmol/L Tris (pH 7.5), 7.5 mmol/L MgCl2, and γ-32P-ATP.

Mouse tumor studies
MDA-MB-231-luc-D3H2LN cells were transduced with control or B55δ short hairpin RNA (shRNA)-expressing lentiviruses, combined with Matrigel (BD Biosciences), and 2 × 106 cells injected into the mammary fat pads of 4- to 6-week-old female beige nude mice. Twelve mice were injected for each group. Tumor growth and metastasis were monitored using a Xenogen IVIS 200 Imaging System.

Statistical analysis
Linear and nonlinear regression analyses were used to evaluate potential correlations between expression of PP2A β-subunits, Fbxw7/α, and CCNE1 and cyclin E1 protein levels in breast tumors and differences in cell and tumor growth rates, respectively. A P value <0.05 was considered statistically significant. All calculations were performed using GraphPad Prism 5 software (GraphPad Software Inc.).

Results
PP2A dephosphorylates cyclin E1 and protects it from ubiquitylation by SCFFbxw7
Because cyclin E1 degradation is triggered by Cdk2 and GSK3 phosphorylations, we explored whether cyclin E1 overexpression in cancers could possibly be caused by dysregulation of a protein phosphatase that counteracted these activities. Cyclin E1 and components of protein phosphatase 2A (PP2A) were previously identified in immunocomplexes of DNA polymerase α-primase (19). Interestingly, PP2A had been previously shown to regulate the phosphorylation status and degradation of c-Myc, another SCFFbxw7 substrate (20, 21). PP2A is a Ser/Thr phosphatase, which plays important roles in cell division control and cancer. We confirmed that both expressed and endogenous cyclin E1 interacted with the α (structural) and C (catalytic) subunits of PP2A by immunoprecipitation (IP)–Western blot analysis (Fig. 1A and B). Incubation of recombinant cyclin E1-Cdk2 complexes with recombinant PP2A core enzyme showed that PP2A could dephosphorylate both the N- and C-terminal phosphodegrons of cyclin E1 (Fig. 1C). In support of this in vitro result, knockdown of Cα subunit expression in HeLa cells by siRNA was found to induce significant increases in phosphorylation of both Thr77 and Thr395 of cyclin E1 (Fig. 1D). These data suggested that PP2A might function to protect cyclin E1 from ubiquitylation by counteracting Cdk2- and GSK3-mediated phosphorylations. To test this, we coexpressed cyclin E1, Cdk2, Fbxw7/α, and HA-ubiquitin in HEK293T cells and then knocked down Cα expression by siRNA. IP–Western blot analysis revealed that Cα knockdown significantly increased ubiquitylation of cyclin E1 in vivo (Fig. 1E). However, no increase in ubiquitylation was observed in similar experiments that used a mutant version of cyclin E1 that had both its phosphodegrons mutated (Thr77Ala/Thr395Ala), confirming the effect was mediated through cyclin E1 phosphorylation (Fig. 1E). Furthermore, addition of PP2A core enzyme to in vitro ubiquitylation

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reactions of cyclin E1 by SCF<sup>Fbw7</sup> was found to decrease the ubiquitylation efficiency (Fig. 1F). Collectively, these results indicated that PP2A counteracted cyclin E1 phosphorylations, thus protecting it from ubiquitylation by SCF<sup>Fbw7</sup>.

**PP2A-B55β regulates cyclin E1 phosphorylation**

PP2A exists as a dimeric core composed of A and C subunits bound to a regulatory (B) subunit, which dictates subcellular localization and substrate specificity (22). Humans express 2-A, 2-C, and 16-B subunits, allowing for a diverse array of enzyme compositions, each with presumably a unique set of substrates. Ectopic coexpression experiments revealed that cyclin E1 coprecipitated with several subunits of the B55 and B56 β-subunit families in vivo (Fig. 2A). Knockdown of expression of these β-subunits in HeLa cells showed that B55β and B56β, and to a lesser extent B56β, reduced cyclin E1 protein levels comparable with Cα knockdown (Fig. 2B), as would be expected if their loss of function deprotected cyclin E1 from ubiquitin-dependent proteolysis. Several lines of evidence led us to exclude both B56β and B56δ as potential regulators of cyclin E1. First, we could not verify an in vivo interaction between these β-subunits and endogenous cyclin E1 protein (Supplementary Fig. S1). Second, quantitative PCR (qPCR) analysis failed to detect any correlation between B56β expression and cyclin E1 protein levels in breast cancer specimens (described below). Third, the expression of both B56δ and B56δ during the cell division cycle was not consistent with a potential role in cyclin E1 degradation, with both peaking in S-phase when cyclin E1 degradation is maximal (see Fig. 3B). In addition, mass spectrometry analysis of B55β...
identified several β-subunits as potential interacting proteins, suggesting the observed interactions between B56β or B56δ and cyclin E1 could be indirect (data not shown). We therefore focused on B55β, encoded by PPP2R2B, and its potential regulation of cyclin E1. Knockdown of B55β expression using three different siRNAs confirmed a reductive effect on cyclin E1 protein level, even though CCNE1 expression and cell cycle kinetics were not adversely affected (Supplementary Figs. S2 and S3). B55β was found to coprecipitate with endogenous cyclin E1 protein (Fig. 2C). Cell fractionation experiments also showed that B55β localized to both the cytoplasm and nucleus in HeLa cells, mirroring cyclin E1 (Fig. 2D). This result was contrary to previous reports, which found that B55β is localized to the cytoplasm (23, 24). In addition, treatment of cells with proteasome inhibitor MG132 or chemical inhibitors of Cdk2 and GSK3 kinases was found to diminish the reduction in cyclin E1 caused by B55β knockdown, confirming that the effect of B55β on cyclin E1 was both proteasome- and phosphorylation-dependent (Fig. 2E and F). Moreover, PP2A-B55β complexes assembled in HEK293T cells were found to efficiently dephosphorylate both the N- and C-terminal phosphodegrons of recombinant cyclin E1 in vitro. PP2A-B55β complexes were used in in vitro phosphatase reactions with recombinant cyclin E1-Cdk2 as substrate.

**PP2A-B55β regulates cyclin E1 phosphorylation.**

A, cyclin E1 co-IPs with B-regulatory subunits B55β, B55δ, B56β, and B56δ in HEK293T cells. B, knockdown of B55β, B56β, and B56δ expression in HeLa cells decreases cyclin E1 protein levels. Ku86 is shown as a loading control. C, endogenous cyclin E1 co-IPs with Flag-B55β expressed in HeLa cells. D, B55β is localized to the cytoplasm and nucleus in cells. HeLa cells were transfected with a plasmid that expresses Flag-B55β and cytoplasmic and nuclear fractions isolated. Exclusive nuclear protein PARP is shown as verification of fractionation efficiency. E, B55β knockdown-induced decrease of cyclin E1 in HeLa cells is proteasome-dependent. B55β expression was knocked down for 48 hours and proteasome inhibitor MG132 added 4 hours before harvesting cells. F, B55β knockdown-induced decrease of cyclin E1 is phosphorylation-dependent. Cells were treated with Cdk2 and GSK3 inhibitors for 4 hours before harvesting. G, PP2A-B55β dephosphorylates cyclin E1 in vitro. PP2A-B55β complexes were assembled in HEK293T cells and used in in vitro phosphatase reactions with recombinant cyclin E1-Cdk2 as substrate.

**PP2A-B55β regulates cyclin E1 stability and determines its level in cell division cycles and cancer-derived cell lines**

We next evaluated the potential importance of PP2A-B55β in determining cyclin E1 levels in cellular division and cancer. Cyclohexamide half-life determination experiments showed that B55β expression significantly increased the stability of cyclin E1 expressed in HEK293T cells (20 vs. >90 minutes;
levels in cancer cells were largely determined by PP2A-B55 accumulation (Fig. 3C). These results suggested that cyclin E1 protein level, its ectopic expression induced cyclin E1 hyperphosphorylation, whereas cyclin A and B1 levels were unaffected (Fig. 3B). In addition, qPCR analysis revealed B55β modulation of B55β required for its degradation (e.g., Fbxw7 and Cdk2), and then expression was enhanced for breast cancers that expressed CCNE1 at ≥50% above the mean (R² = 0.70 vs. 0.10; Fig. 4B). Because cyclin E1 degradation is regulated by SCF<Fbxw7>, we also analyzed its potential influence on determining cyclin E1 levels in breast cancers. qPCR analysis failed to uncover a significant correlation between cyclin E1 protein level and FBXW7a expression in these specimens (Supplementary Fig. S4). Furthermore, our previous analysis showed that these same breast cancer specimens do not contain mutations of the FBXW7 gene, a potential mechanism of SCF<Fbxw7> inactivation in cancers (16). Of note, we also found no correlations between cyclin E1 protein levels and expression of B56δ or B56δ (Supplementary Fig. S4). Therefore, these data demonstrated that the level of cyclin E1 in cancer cells is largely determined by B55β.

PP2A-B55β inhibition reduces breast cancer cell growth in vitro and tumor formation in vivo

We next tested the potential therapeutic application of PP2A-B55β inhibition as a means of decreasing cyclin E1 levels in breast cancer cells. Knockdown of B55β expression in a panel of breast cancer cell lines representing both luminal hormone receptor–positive (ER⁺, PR⁺; MCF-7, and T47-D) and basal-like TNBCs (ER⁺, PR⁺, Her2⁺; BT-549, MDA-MB-231, MDA-MB-468, MDA-MB-157, MDA-MB-436, and SUM149PT) was found to decrease cyclin E1 protein levels in all cell lines except SUM149PT (Fig. 4C), which has been shown to harbor...
FBXW7 gene mutations (25). Of note, B55β knockdown even reduced cyclin E1 levels in MDA-MB-157 cells, which contain a CCNE1 gene amplification and overexpress cyclin E1 at 64-fold the level of normal breast epithelial cells (26). B55β expression knockdown also resulted in reduced cyclin E1-Cdk2–associated kinase activity (Fig. 4D). To evaluate the effects of B55β expression knockdown on breast cancer cell growth, we transduced two basal-like TNBC cell lines (MDA-MB-231 and MDA-MB-157) with B55β shRNA-expressing or control lentiviruses. B55β knockdown was found to significantly reduce long-term cell proliferation (Fig. 4E). Moreover, MDA-MB-231-luc cells transduced with B55β shRNA-expressing lentiviruses displayed a significantly reduced growth rate and ability to form tumors when implanted into the mammary fat pads of nude mice compared with control cells (Fig. 5A and B). The B55β shRNA-expressing tumors also failed to metastasize to bronchial lymph nodes (0/12 vs. 2/12 for control tumors; Fig. 5C), the preferred metastatic site for this model system. These

Figure 4. B55β expression correlates with cyclin E1 levels in breast cancers and its knockdown inhibits breast cancer cell proliferation. A and B, B55β expression correlates with cyclin E1 protein level in breast cancers. PP2R2B and CCNE1 expressions were quantified by qPCR and cyclin E1 protein by Western blot analysis. A, all tumors. B, breast cancer specimens that express CCNE1 at >50% above the mean. C, B55β expression knockdown reduces cyclin E1 levels in a panel of breast cancer cell lines with functional SCFFbxw7. qPCR data showing B55β knockdown efficiency are shown below. Cell line SUM149PT contains defective FBXW7 alleles. D, B55β knockdown reduces cyclin E1–associated kinase activity in breast cancer cells. Cyclin E1-Cdk2 was immunoprecipitated from extracts and used in in vitro kinase assays with human recombinant pRb as substrate. E, B55β knockdown inhibits breast cancer cell proliferation. TNBC cell lines MDA-MB-231 and MDA-MB-157 were transduced with control or B55β shRNA-expressing lentiviruses and cells counted. Experiments were performed in triplicate.
results demonstrated that B55β inhibition can efficiently reduce cyclin E1 levels in breast cancer cells, and limits their growth and tumorigenicity.

Discussion

Although cyclin E1 overexpression is an important prognostic marker in breast and other cancers, the molecular cause(s) of these alterations was poorly understood. Our data show that the protein phosphatase PP2A-B55β regulates cyclin E1 levels in cellular division cycles and contributes to its dysregulation in cancers (Fig. 5D). During G1 phase, PP2A-B55β antagonizes phosphorylations of cyclin E1 mediated by autophosphorylation and GSK3 kinase, allowing for cyclin E1 to accumulate and initiate an S-phase program. After S-phase is initiated, B55β levels decline, which deprotects cyclin E1 from phosphorylation, thus triggering its recognition and ubiquitylation by SCF^Fbxw7^7. In support of a possible involvement of PP2A in cyclin E1 regulation, a recent report found that in yeast PP2A^Cdc55^ controls the stability of G1 cyclin Cln2 through regulation of its phosphorylation state (27). In cancers, this regulation can be aberrant and augmented B55β expression functions to antagonize cyclin E1 degradation, leading to its hyperaccumulation especially in cells that overexpress CCNE1. The overexpressed cyclin E1 then promotes cancer cell survival, hormone-independent growth, genetic instability, and progression to advanced disease.

The ability of augmented B55β to promote cyclin E1 overexpression suggests that it could function as an oncoprotein in human tumorigenesis. Indeed, a search of the ONCOMINE database revealed that PPP2R2B is overexpressed in several cancer types, including breast, ovary, brain, and liver.

Figure 5. B55β knockdown inhibits breast cancer formation in nude mice and model of PP2A-B55β regulation of cyclin E1 in cellular division and cancer. A, growth curves of control and B55β shRNA-expressing breast cancers. MDA-MB-231-luc-D3H2LN cells were transduced with control or B55β shRNA-expressing lentiviruses and then implanted into the mammary fat pads of nude mice. Twelve mice were analyzed for each cell line. B, representative luminescent images of tumors 6 weeks postimplantation. Examples of excised tumors at 8 weeks postimplantation are shown on right. C, B55β knockdown inhibits metastasis progression. Luminescent images of mice 8 weeks postimplantation showing bronchial metastases of a control cell tumor but not B55β shRNA-expressing tumor. D, model of B55β regulation of cyclin E1 in cellular division and cancer. Details explained in text.
(Supplementary Fig. S5). However, a previous report showed that PPARγ2 is often transcriptionally repressed by promoter hypermethylation in colorectal cancers, suggesting it functions as a tumor suppressor in these cancers (29). Contrary to this study though, we found that knockdown of B55β expression in colorectal cancer cell lines HT29 and HCT116, which contain hypermethylated PPARγ2 alleles, significantly reduced their proliferation and cyclin E1 levels (Supplementary Fig. S6). A possible explanation for this discrepancy could be that reduced B55β expression imparts tumorogenic properties on colorectal cancer cells that are independent of cyclin E1, though B55β expression is indispensable for proliferation.

PP2A-B55β ablation reduced cyclin E1 protein levels in breast cancer cells that contain functional SCF<sup>FBw7</sup>, suggesting its inhibition could represent a rational therapeutic strategy for inactivating cyclin E1 function in cancers. The oncogenic potential of cyclin E1 has been shown to be mediated through both Cdk2-dependent and -independent functions, the latter of which includes promotion of G<sub>1</sub>-S-phase transition and oncogene-mediated transformation (30). Therefore, PP2A-B55β inhibition could represent a more effective therapeutic mechanism for abrogating cyclin E1 functions in cancers than drugs that target Cdk2 kinase activity, because both Cdk2-dependent and -independent functions would be affected. PP2A-B55β-directed therapies might be particularly effective for treatment of highly aggressive basal-like TNBCs, which typically overexpress cyclin E1, lack targeted and effective therapies, and whose growth and survival have been shown to depend on these abnormalities (31). They might also be effective as a prophylactic treatment for carriers of BRCA1 mutations, who exhibit an increased risk of developing basal-like TNBCs in their lifetime.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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