The Prolyl Isomerase Pin1 Acts Synergistically with CDK2 to Regulate the Basal Activity of Estrogen Receptor α in Breast Cancer

Chiara Lucchetti¹, Isabella Caligiuri¹,²,³, Giuseppe Toffoli⁴, Antonio Giordano¹,²,³, Flavio Rizzolio¹,⁴*

¹Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia, Pennsylvania, United States of America, ²Human Health Foundation, Terni and Spoleto (PG), Italy, ³Department of Human Pathology and Oncology, University of Siena, Siena (SI), Italy, ⁴Division of Experimental and Clinical Pharmacology, Department of Molecular Biology and Translational Research, National Cancer Institute and Center for Molecular Biomedicine, Aviano (PN), Italy

Abstract

In hormone receptor-positive breast cancers, most tumors in the early stages of development depend on the activity of the estrogen receptor and its ligand, estradiol. Anti-estrogens, such as tamoxifen, have been used as the first line of therapy for over three decades due to the fact that they elicit cell cycle arrest. Unfortunately, after an initial period, most cells become resistant to hormonal therapy. Peptidylprolyl isomerase 1 (Pin1), a protein overexpressed in many tumor types including breast, has been demonstrated to modulate ERalpha activity and is involved in resistance to hormonal therapy. Here we show a new mechanism through which CDK2 drives an ERalpha-Pin1 interaction under hormone- and growth factor-free conditions. The PI3K/AKT pathway is necessary to activate CDK2, which phosphorylates ERalphaSer294, and mediates the binding between Pin1 and ERalpha. Site-directed mutagenesis demonstrated that ERalphaSer294 is essential for Pin1-ERalpha interaction and modulates ERalpha phosphorylation on Ser118 and Ser167, dimerization and activity. These results open up new drug treatment opportunities for breast cancer patients who are resistant to anti-estrogen therapy.

Citation: Lucchetti C, Caligiuri I, Toffoli G, Giordano A, Rizzolio F (2013) The Prolyl Isomerase Pin1 Acts Synergistically with CDK2 to Regulate the Basal Activity of Estrogen Receptor α in Breast Cancer. PLoS ONE 8(2): e55355. doi:10.1371/journal.pone.0055355

Editor: Karin Dahlman-Wright, Karolinska Institutet, Sweden

Received August 21, 2012; Accepted December 27, 2012; Published February 4, 2013

Copyright: © 2013 Lucchetti et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) (A.G.), Special Program Molecular Clinical Oncology, 5 × 1000, (number 12214) (G.T.), European Research Council, Programme “Ideas”, proposal number 269051 (F.R., G.T.), Italian Ministry of Education MIUR (FIRB prot. RBAP11ETKA (G.T.)), Sbarro Health Research Organization (A.G.), Istituto Tumori Toscano ITT (A.G.), Human Health Foundation (A.G.) and Commonwealth of Pennsylvania (A.G.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

In the normal mammary gland, estrogen receptor alpha (ERalpha) and its ligand, estradiol (E2), primarily control the ductal outgrowth and side branching that occur during pregnancy and the menstrual cycle [1,2]. Only 15–25% of mammary epithelial cells express ERalpha. These cells appear to be in a non-proliferative state and act to stimulate the growth of surrounding ERalpha-negative cells in response to estrogen. By contrast, the majority (70%–80%) of primary breast tumors express high levels of ERalpha and the growth of these tumors is estrogen-regulated [3]. As a consequence, inhibition of ERalpha activity by hormonal therapies reduces recurrence and improves clinical outcomes in breast cancer patients [4,5].

ERalpha belongs to the superfamily of steroid nuclear receptor transcription factors and shares four main functional domains with other nuclear hormone-responsive receptors: the N-terminal transactivation function (AF-1) domain, DNA-binding domain (DBD), hinge region and ligand-binding domain (LBD) (AF-2). When estradiol binds to the AF-2 domain, it induces structural changes that facilitate ERalpha dimerization, nuclear translocation and the binding of DNA to regulate gene transcription [6]. The AF-1 domain is regulated by growth factor receptor signaling, including epidermal growth factor receptor (EGFR), HER2 and insulin-like growth factor receptor (IGF1-R) [7]. The DBD and LBD domains are structurally ordered with a globular profile when expressed independently [8]. The three-dimensional native-fold structure of the N terminal and hinge domains has not yet been resolved as they are intrinsically disordered regions [9].

The hinge region, although initially characterized as merely a flexible connection between the DBD and LBD regions, has recently been discovered to have a more complex function. Indeed, it comprises the nuclear translocation signal and includes estrogen-independent regulatory sequences: selective mutation studies showed that the binding of ERalpha to c-Jun and Sp-1 transcription factors requires an intact hinge domain [10]. The complexity of this domain is further increased since its functional transactivation is mediated by many posttranslational modifications such as methylation, acetylation, sumoylation and phosphorylation [11–13]. In particular, the hinge region contains Ser294, a canonical Ser/Thr-Pro motif. This motif has been discovered to have a pivotal function in the regulation of protein activity.

The Ser/Thr-Pro motifs exist in two distinct conformations: cis and trans [14]. Significantly, phosphorylation on Ser/Thr-Pro motifs has a steric hindrance function that further restrains the already slow cis/trans prolyl isomerization of peptide bonds [15]. Peptidylprolyl isomerase 1 (Pin1) is a unique member of one out of three protein families, the parvulins, which interacts with and...
isomerizes phosphorylated Ser/Thr-Pro motifs. The final result of the isomerization activity of Pin1 is a conformational change that can alter protein function, localization and/or stability [16]. For the aforementioned reasons, Pin1 has multiple roles in tumorigenesis [17] with important implications in breast cancer development and resistance to hormonal therapy [18–22]. With regards to ERalpha, it has recently been reported that Pin1 can directly and indirectly regulate its activity. Pin1 can stimulate the function of ERalpha by promoting SRC-3 coactivator activity and turnover [23] or by promoting CDK2-dependent SMRT corepressor protein degradation [24]. Finally, it has been demonstrated that Pin1 can directly interact with EBs- or estrogen-stimulated ERalpha, regulating its transcriptional activity through Ser118 [4]. ERalpha, regulating its transcriptional activity through Ser118 [4].

In the present study, we show that Pin1 regulates the functions of ERalpha under basal conditions through its interaction with Ser294. We demonstrate that a mutation of Ser294 reduces the levels of Ser118 and Ser167 phosphorylation with important implications for ERalpha dimerization and activation. Finally, we propose a new working model for Pin1 and ERalpha and suggest a novel pathway that can be inhibited to overcome resistance to hormonal therapy.

**Materials and Methods**

**Cells culture conditions**

MCF7 breast cancer cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). 293FT cells were grown at 37°C, in a 5% CO2/95% atmosphere. Hormone-free medium was prepared with phenol red-free EMEM with 5% charcoal-stripped FBS.

**Reagents**

Antibodies were purchased from: Pin1 (600-401-A20), 6XHis (600-410-382), from Santa Cruz Biotechnology, Santa Cruz, CA, USA; α-tubulin (T-6074) from Sigma Inc., St Louis, MO, USA; AKT (9272), MAPK (9102), p-MAPK (9101) from Cell Signaling, Beverly, MA, USA. Kinase inhibitors were purchased from: flavopiridol (F9053) from Sigma Inc., St Louis, MO, USA; AG825 (1553) and U0126 (1144) from Tocris Bioscience, Ellisville, Missouri 63021, USA; LY294002 (9901) from Cell Signaling, Beverly, MA, USA. Estradiol was purchased from Sigma Inc., St Louis, MO, USA.

**Plasmids**

shRNA plasmids Pin1 (SHCLNG-NM_006221), CDK1 (SHCLNG-NM_001786), CDK2 (SHCLNG-NM_001798) and CDK4 (SHCLNG-NM_000075) were obtained from Sigma Inc., St Louis, MO, USA. Scrambled shRNA (17920), pPAX2 packaging plasmid (12260) and pMDG-2 envelope plasmid (12259) were obtained from Addgene Inc, Cambridge, MA, USA.

**GST pull-down assay**

GST and GST-Pin1 proteins were produced in BL21 bacteria cells. Cells were grown to mid log phase and then induced to express protein by adding 0.25 mM of isopropyl-1-thio-D-galactopyranoside (IPTG, Roche Applied Science, Indianapolis, IN, USA). The cultures were shaken for 4 h; bacteria were then pelleted and resuspended in NENT buffer (20 mM Tris (pH 8), 50 units/mL penicillin, 50 μg/mL streptomycin, and 3% charcoal-stripped FBS.

**Real-time PCR**

Total RNA was prepared from tissues using the RNA extraction kit RNAeasy (Qiagen Inc, Valencia, CA, USA). One μg of total RNA was reverse transcribed in a 20 μl reaction using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Primers to amplify CTSD, TFF1, GPDH were as follows: CTSD-I 5'-GCT GGC ACG CCA ACG GGC CGC-3' and ERS294A-r 5'-GCC TTT GAT CAT CGA ATG TGG-3' and ERS294A-r 5'-GCC TTT GAT CAT CGA ATG TGG-3'. All the plasmids were sequenced for accuracy.

**Lentiviral production**

To generate knock down cells, lentiviral particles were produced as described (http://www.broadinstitute.org/genome_bio/trc/publicProtocols.html) and Rizzolio et al. [30].

Pin1 Regulates ERα

ERalpha-His-AB, His-CD and His-EF plasmids were derived from VP16-ERalpha (ADDGENE:11351) following amplification with primers: AB-BamHI-F ATG GAT CCA CCA TGA CCA TGA CCA CCC TCC-3', ERalphaSer118, Ser167, and to a lesser extent, Ser104/106, are the main residues phosphorylated in the AF-1 domain [25–29] and have a central role in the regulation of transcriptional activity of the receptor [25].
Co-immunoprecipitation assay

Sub-confluent MCF7 cells were harvested and proteins were prepared as follows: the cell pellet was resuspended in lysis buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% NP40, 2 mM EDTA). 1 mg of proteins was immunoprecipitated, utilizing 4 μg of Pin1, ERalpha antibody or mouse IgG overnight at 4°C. The immunoprecipitated protein complex was collected with agarose protein A/G beads (Pierce) for 3 h at 4°C. After washing, the protein immunocomplex was run on SDS-PAGE followed by immunoblot analyses to detect Pin1 or ERalpha proteins.

In vitro kinase assays

GST-D WT or mutant constructs were purified from E. Coli, 1 μg of recombinant protein incubated with 100 U of CDK2/CyclinA, CDK4/CyclinD1, CDK6/CyclinD1 (SigmaChem, Richmond, Canada) in the kinase reaction buffer (40 mM Tris HCl, 20 mM MgCl2, 0.1 mg/ml BSA, 0.2 mM, ATP, 2 mM DTT) for 30 minutes at RT. The reaction was analyzed with the Kinase Glo luminescence assay (Promega, Wisconsin, USA) according to the manufacturer’s protocol.

Results

Pin1 interacts with ERalpha under basal conditions

Previous studies demonstrated that Pin1 indirectly controls ERalpha activity [23,24] or via direct binding to phosphorylated Ser118 under hormone or growth factor stimulation [4]. To further clarify the role of Pin1 in the regulation of ERalpha activity, we immunoprecipitated Pin1 in the presence or absence of 17β-estradiol in MCF7 cells (Fig. 1A). Pin1 kd cells (Fig. 1B) and IgG were used as a negative control. We demonstrated that, under both conditions, Pin1 forms a macromolecular complex with ERalpha in vitro. To understand which mechanism is involved in the interaction between Pin1 and ERalpha under basal conditions, we then decided to carry out all experiments in the absence of FBS and estradiol unless indicated.

Pin1 is a short protein that contains two known functional domains, a WW interacting domain (amino acids 6–37) and a PPlase domain (amino acids 54–163). Total cellular lysate was incubated with GST, GST-Pin1 protein, GST-WW and GST-PP domains (Fig. 1C). GST pull-down assays confirmed that both the WW domain and PPlase domain are critical for ERalpha interaction.

The CD domain in ERalpha is responsible for Pin1 binding

To clarify which ERalpha domain is involved in the binding of Pin1, ERalpha protein was split into three regions (domains A/B, CD and EF) (Fig. 2A) and fused with a C-terminal Histidine tag for further analysis. Plasmids were transfected in 293FT cells and total cellular lysate was pulled down with GST and GST-Pin1 proteins (Fig. 2B). Using a 6×HIS antibody, we were able to demonstrate that only CD peptide interacts with GST-Pin1 but not GST. As Pin1 is a peptidyl-prolyl-isomerase that specifically recognizes and isomerizes phosphorylated Ser/Thr-Pro motifs, we explored putative sites in the CD region that match the consensus motif. We found that Ser294 was a unique Ser-Pro site located in the D domain (Fig. 2A). To confirm that Ser294 is responsible for the binding between ERalpha and Pin1, a Ser294Ala mutation (MT) was introduced by site-directed mutagenesis. 293FT cells were transfected with CD wild-type and mutant domains. GST-pull
a bait protein to immunoprecipitate the CD domain. After pull down, we observed a reduced amount of the CD domain in 293FT cells expressing shRNA CDK2 compared to shRNA CDK1 or CDK4 proteins (Fig. 3B). To confirm this result, a kinase assay was performed on WT and Ser294Ala D domains and used as a substrate for the active form of CDK2/CycA, CDK4/CycD1 and CDK6/CycD1 kinase complexes. Detection of kinase activity was performed using Kinase Glo luminescence assay, CDK2 efficiently phosphorylates the wild-type D domain and to a lesser extent the Ser294Ala D domain. CDK4 and CDK6 show a very weak activity on the D domain. The result confirms that CDK2 can bind and phosphorylate ERalpha in the D domain and facilitates Pin1 binding by generating a phospho-Ser-Pro site (Fig. 3C).

In order to further demonstrate the involvement of the CDK pathway in Pin1-ERalpha binding, we decided to use flavopiridol (FLV), a potent CDKs-inhibitor. MCF7 cells cultured in phenol-red-free and hormone-free medium were treated for 16 hours with 0.25-0.125 μM concentrations of FLV. As expected, inhibition of CDK activity blocked Pin1 binding on ERalpha (Fig. 3D).

Ser294 is involved in Ser118 and Ser167 phosphorylation and participates in ERalpha dimerization

Phosphorylation of ERalpha on serine 118 and 167 is required for the full activity of protein function [25]. Both residues are located within the N-terminal region (AF-1), which is known to promote transcription in a ligand-independent manner. However, phosphorylation of these serines is enhanced in response to ligand binding in the AF-2 domain. In order to examine the significance of Ser294 in relation to ERalpha phosphorylation in vivo, a full-length ERalpha expression plasmid bearing the mutation Ser294Ala was generated. 293FT cells were co-transfected with ERalpha WT or Ser294Ala and GFP plasmid as controls. Two days before treatment, cells were grown in phenol-red-free medium containing 3% charcoal-stripped FBS. After 45 min of E2 treatment, cellular pellets were collected and lysed for Western blot analysis. We could note that phosphorylation on Ser118 and Ser167, both implicated in ERalpha transcriptional activation, was reduced in cells transfected with the Ser294Ala ERalpha plasmid. The Ser294Ala is located in the D domain, a flexible region that lies between two distinct dimerization interfaces in the DBD and LBD regions [34]. To investigate the involvement of Ser294 in E2-dependent ERalpha dimerization and transcriptional activation, a mammalian two-hybrid assay was used. ERalpha-WT and Ser294Ala constructs were cloned in pGAL4 (DBD) and pVP16 to generate hybrid fusion proteins and transfected as homodimers in 293FT cells. Transfected cells were treated with E2 or a vehicle for 16 h. We observed a statistically significant reduction of ERalphaSer294Ala activity compared to wild-type ERalpha following hormone-induced stimulation.

To confirm that Pin1 modifies ERalpha activity, we carried out a real-time PCR (qRT-PCR) on two well-known estrogen-induced genes (CTSD and TFF1) in Pin1 kd MCF7 cells treated with E2 for 6 h (Fig. 4c). We were able to detect a reduction in mRNA levels for both the CTSD and TFF1 genes.

**Figure 2.** ERalpha CD domain interacts with Pin1. A) Schematic representation of ERalpha domains, showing the predicted prolyl isomerase sites (Ser/Thr-Pro). B) ERalpha cDNA was split into three different domains (AB, CD, EF) and tagged with histidine. GST or GST-Pin1 pull-down assay was performed in 293FT cells and analyzed by Western blot with anti-6xHis antibody. C) Site-directed mutagenesis was performed to replace Serine 294 with Alanine on the ERalphaCD domain. Plasmids with wild-type (w) or mutant (m) CD His-tagged domain were transfected in 293FT cells and pulled down with GST-Pin1 antibody. Anti-His antibody was used in Western blots to detect the interaction.

doi:10.1371/journal.pone.0055355.g002

down experiments showed that mutation in the CD peptide greatly reduced the ability of the ERalpha protein to bind to Pin1, (Fig. 2C) confirming Ser294 as the Pin1 binding site.

CDK2 interacts with and phosphorylates the CD peptide to generate the Pin1-binding site

Since Pin1 activity requires prior phosphorylation of its target site and the Ser/Thr-Pro motif is the minimal consensus sequence recognized by Cyclin Dependent Kinases (CDKs) [35], we investigated whether CDK-mediated phosphorylation of Ser294 elicits Pin1 binding. For this purpose, shRNA CDK1, CDK2 and CDK4 were stably expressed in 293FT cells (Fig. 3A) and transfected with the ERalpha CD domain. GST-Pin1 was used as a bait protein to immunoprecipitate the CD domain. After pull down, we observed a reduced amount of the CD domain in 293FT cells expressing shRNA CDK2 compared to shRNA CDK1 or CDK4 proteins (Fig. 3B). To confirm this result, a kinase assay was performed on WT and Ser294Ala D domains and used as a substrate for the active form of CDK2/CycA, CDK4/CycD1 and CDK6/CycD1 kinase complexes. Detection of kinase activity was performed using Kinase Glo luminescence assay, CDK2 efficiently phosphorylates the wild-type D domain and to a lesser extent the Ser294Ala D domain. CDK4 and CDK6 show a very weak activity on the D domain. The result confirms that CDK2 can bind and phosphorylate ERalpha in the D domain and facilitates Pin1 binding by generating a phospho-Ser-Pro site (Fig. 3C).

In order to further demonstrate the involvement of the CDK pathway in Pin1-ERalpha binding, we decided to use flavopiridol (FLV), a potent CDKs-inhibitor. MCF7 cells cultured in phenol-red-free and hormone-free medium were treated for 16 hours with 0.25-0.125 μM concentrations of FLV. As expected, inhibition of CDK activity blocked Pin1 binding on ERalpha (Fig. 3D).

Ser294 is involved in Ser118 and Ser167 phosphorylation and participates in ERalpha dimerization

Phosphorylation of ERalpha on serine 118 and 167 is required for the full activity of protein function [25]. Both residues are located within the N-terminal region (AF-1), which is known to promote transcription in a ligand-independent manner. However, phosphorylation of these serines is enhanced in response to ligand binding in the AF-2 domain. In order to examine the significance of Ser294 in relation to ERalpha phosphorylation in vivo, a full-length ERalpha expression plasmid bearing the mutation Ser294Ala was generated. 293FT cells were co-transfected with ERalpha WT or Ser294Ala and GFP plasmid as controls. Two days before treatment, cells were grown in phenol-red-free medium containing 3% charcoal-stripped FBS. After 45 min of E2 treatment, cellular pellets were collected and lysed for Western blot analysis. We could note that phosphorylation on Ser118 and Ser167, both implicated in ERalpha transcriptional activation, was reduced in cells transfected with the Ser294Ala ERalpha plasmid. The Ser294Ala is located in the D domain, a flexible region that lies between two distinct dimerization interfaces in the DBD and LBD regions [34]. To investigate the involvement of Ser294 in E2-dependent ERalpha dimerization and transcriptional activation, a mammalian two-hybrid assay was used. ERalpha-WT and Ser294Ala constructs were cloned in pGAL4 (DBD) and pVP16 to generate hybrid fusion proteins and transfected as homodimers in 293FT cells. Transfected cells were treated with E2 or a vehicle for 16 h. We observed a statistically significant reduction of ERalphaSer294Ala activity compared to wild-type ERalpha (Fig. 4B). These experiments show that phosphorylation of Ser294 is required for proper phosphorylation of ERalpha following hormone-induced stimulation.

To confirm that Pin1 modifies ERalpha activity, we carried out a real-time PCR (qRT-PCR) on two well-known estrogen-induced genes (CTSD and TFF1) in Pin1 kd MCF7 cells treated with E2 for 6 h (Fig. 4c). We were able to detect a reduction in mRNA levels for both the CTSD and TFF1 genes.

**P38/AKT is involved in the Pin1-ERalpha interaction**

ERalpha is regulated by multiple signaling pathways including the P38/AKT pathway and the extracellular-regulated kinase pathway (MAPK). Neither pathway excludes the other, and they can activate ERalpha in a ligand-dependent or -independent manner [25]. To investigate which pathways stimulate the Pin1-ERalpha interaction, we treated the MCF7 cells with drug
inhibitors of PI3K (LY294002), Her2 (AG825) and Mek1/2 (U0126). We performed the experiments in the absence or presence of FBS, with similar results. GST-pull down with Pin1 fusion protein performed on MCF7 cellular lysates showed a marked reduction in Pin1/ERα binding in the presence of the PI3K/AKT inhibitor LY294002 (Fig. 5A and data not shown). These results suggest a contribution of the PI3K/AKT pathway and CDK2 activity on ERαSer294. Differently, Her2 and Mek1/2 inhibitors did not affect Pin1 binding, excluding their involvement in this mechanism (data not shown).

Discussion

Evaluation of the ERα status is an essential component in the pathological classification of breast cancers and determines the success of endocrine therapies [35]. Endocrine agents are currently used as first line therapy for ERα-positive breast cancers [36]. These treatments target the ERα pathway by blocking the receptor's activity or starving the tumor of estrogens.

A number of studies suggest that the benefit of endocrine therapy is proportional to the level of ERα expression [37–39] and therefore further characterization of the factors that determine the precise levels of ERα is essential. However, although beneficial, resistance to hormonal therapy occurs in 50% of patients and it remains crucial to determine the underlying molecular mechanism [40]. Recent data support the concept that Pin1 is implicated in the acquired resistance to tamoxifen [4,18,41], a drug that has been used in the clinical setting for more than 30 years [7]. In particular, Pin1 is involved in the overexpression of the HER-2 receptor [18] and controls the activity of ERα by regulating the stability of its cofactors, SRC-3 [23] and SMRT [24]. Furthermore, it has been demonstrated that under FBS or estradiol conditions, Pin1 can regulate ERα activity through interaction with phospho-Ser118 [4]. The authors suggested that when Ser118 is phosphorylated, the ERα N-terminus is preferentially restricted in the cis conformation. After Pin1 binding, the AF1 domain is switched to the trans conformation by promoting a slight local structural change [4].

Here we report that under basal conditions, ERα Ser294 is the target of Pin1. We demonstrated by GST pulldown and coimmunoprecipitation assays on MCF7 cell extracts that Pin1

---

Figure 3. CDK2 allows ERαSer294 and Pin1 interaction. A) 293FT cells were infected with 1 MOI of specific CDKs or Scrambled shRNA lentivirus as indicated. B) 293FT cells knocked down for CDK1, CDK2 and CDK4 genes were transfected with ERαCD domain and pulled down with GST-Pin1. Quantification represents the ratio between the pull down and total ERα protein and normalized to scrambled cells. C) Kinase assay was done on wild-type or mutant ERαD domains. The D domain was incubated with activated CDK/cyclin complexes as indicated and the kinase activity was measured in Relative Luciferase Unit/Second (RLU/s). Data shown represent average values ± S.D. n = 3. In the lower panel, the GST-D domain and cyclin/CDKs complexes loading controls. D) Cells were treated with flavopiridol at different drug concentrations and pulled down with GST or GST-Pin1. The level of phosphorylation of pRb was used to control the efficacy of flavopiridol.

doi:10.1371/journal.pone.0055355.g003
interacts with ERalpha and this interaction was reduced when serine 294 was mutated to alanine. The hinge region is a relatively short domain spanning 51 amino acids (263–314), and with several important functions in ERalpha regulation. Complete deletion of the hinge region impairs ligand-induced downregulation of the receptor [10,42]. In the N-terminal region (253–282 aa) overlapping the DNA binding domain resides the ERalpha nuclear localization sequence (NLS) that includes a protein-protein interaction sequence (267–275 aa) for c-Jun transcriptional factor [10]. Moreover, sumoylation of lysines 302/303 promotes ligand-induced polyubiquitination, which is required for receptor turnover [43]. The localization of Ser294 in the hinge region near the DBD domain suggests a role [13,44] in DNA binding and/or dimerization of ERalpha. Using a two-hybrid assay, we demonstrated that Ser294 is effectively involved in dimerization and consequently in ERalpha activation. After estradiol stimulation, a significant reduction in transcriptional activity was detected in cells transfected with ERalpha mutant, suggesting that a Ser294Ala mutation impairs dimerization.

On the basis of results in the literature [4] and our own data [45], we proposed a working model in which Pin1 regulates ERalpha in two different steps: under basal conditions, there is a first event mediated by CDK2 phosphorylation that takes place on Ser294. In the second step, growth hormone-dependent transcriptional activity was detected in cells transfected with ERalpha mutant, suggesting that a Ser294Ala mutation impairs dimerization.
factor stimulation promotes Pin1 recruitment to ERα at phosphoSer118, modulating AF-1 functional activity (Fig. 3B). Since the proper phosphorylation levels of Ser118 and Ser167 have been associated with a clinical response of patients treated with tamoxifen [46–48], it appears that Pin1 has a critical role in this mechanism.

Although Ser294 has a proven role in the activity of ERα, there is a lack of information regarding the pathway involved in Ser294 phosphorylation and its functional significance. Serines residues located in the N-terminal domain (Ser104/106, Ser118, Ser167) and in the Hinge region (Ser294) match the consensus sequence recognized by families of Serine/Threonine proline directed kinases, including cyclin-dependent kinases. ERα transcriptional activity is virtually abolished under conditions where CDK2 activity is suppressed by the kinase inhibitor p27 or by a dominant negative CDK2 mutant, regardless the presence or absence of hormones [49]. Thus it appears that CDK2 is a limiting cofactor in the regulation of ERα-dependent transcription. We demonstrated that Ser294 amino acid represents a new target for CDK2, and its phosphorylation is required for Pin1 binding, a mechanism elicited by the PI3K pathway. Currently, the detailed signaling pathway that sustains the Pin1-ERα interaction remains obscure although there are different experimental models, which have demonstrated a link between PI3K and CDK2 activity in a different tissue context. The PI3K pathway promotes Thr-160 phosphorylation in the CDK2 activating site [50–52]. Of note, Pin1 can stabilize the AKT protein [52], suggesting a multiple level mechanism in which Pin1 controls the PI3K/AKT pathway [53]. Further studies will be done to clarify the PI3K signaling cascade in CDK2/ERα regulation in breast cancer.

Overall, our results suggest a new pathway that involves PI3K/ AKT, CDK2 and Pin1 and open up new opportunities for treating ERα-positive breast cancer patients who are resistant to hormonal therapy.

Acknowledgments

We thank Ms. Marie Basso and Mr. Robert Fratamico for their assistance in coping the manuscript.

Author Contributions

Conceived and designed the experiments: CL FR AG. Performed the experiments: CL IC. Analyzed the data: CL FR. Contributed reagents/materials/analysis tools: AG. Wrote the paper: CL FR GT.

References

1. Hemighausen L, Robinson GW (2005) Information networks in the mammary gland. Nat Rev Mol Cell Biol 6: 715–725.
2. Caligiuri I, Rizzolli F, Roflo S, Giorlando A, Toffoli G (2011) Critical choices for modeling breast cancer in transgenic mouse models. J Cell Physiol 227: 2988–2991.
3. Ali S, Balasuwa I, Coombes RC (2011) Anoestrogens and their therapeutic applications in breast cancer and other diseases. Annu Rev Med 62: 217–232.
4. Rajbhandari P, Finn G, Solodin NM, Singarapu KK, Sahu SC, et al. (2012) Regulation of estrogen receptor alpha N-terminus conformation and function by peptidyl prolyl isomerase Pin1. Mol Cell Biol 32: 445–457.
5. Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, et al. (2010) Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 28: 509–518.
6. McDonnell DP, Norris JD (2002) Connections and regulation of the human estrogen receptor. Science 296: 1642–1644.
7. Osborne CK, Schlif R (2011) Mechanisms of endocrine resistance in breast cancer. Annu Rev Med 62: 235–247.
8. Kumar R, Zakharov MN, Khan SH, Jang H, Miki R, et al. (2011) Identification of novel estrogen receptor alpha/estrogen response element gene regulatory mechanisms. J Biol Chem 286: 12640–12649.
9. Senti S, Le Romancer M, Bianchin C, Rostan MC, Corbo L (2005) Suppression of the estrogen receptor alpha hinge region regulates its transcriptional activity. Mol Endocrinol 19: 2671–2684.
10. Wang C, Fu M, Angeletti RH, Siconolfi-Za e L, Reuten AT, et al. (2001) Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transcriptional and hormone sensitivity. J Biol Chem 276: 18375–18383.
11. Williams CG, Basu A, El-Gharbawy A, Carrier LM, Smith CL, et al. (2009) Regulation by phosphorylation of estrogen response element gene regulatory machinery. J Biol Chem 284: 30916–30929.
12. Girardini JE, Polo M, Piazza S, Rustighi A, Marotta C, et al. (2011) A Pin1 mutant p53 axis promotes aggressiveness in breast cancer. Cancer Cell 20: 79–91.
13. Nobili M, Girardini JE, Piazza S, Del Sal G (2011) Wiring the oncogenic circuitry: Pin1 unleashes mutant p53. Oncotarget 2: 654–656.
14. Pi Y, Wu RC, Sandquist J, Wong J, Tsai SY, et al. (2005) Peptidyl-prolyl isomerase I (Pin1) serves as a coactivator of steroid receptor by regulating the activity of phosphorylated steroid receptor coactivator 3 (SRC-3/AIB1). Mol Cell Biol 25: 9687–9699.
15. Stayna KJ, Liu Y, Means AR, Kao HY (2008) CDK2 and Pin1 negatively regulate the transcriptional corepressor SMRT. J Biol Chem 283: 49–61.
16. Lammigan RA (2003) Estrogen receptor phosphorylation. Steroids 68: 1–9.
17. Schiavo, N, Wu, M, Seo, Y, Suen, Y, Wei, J, et al. (2002) Phosphorylation of human estrogen receptor alpha at serine 118 by two distinct signal transduction pathways revealed by phosphorylation-specific antisera.
18. Buoncristiani L, Blandari P, Maistrick RJ, Picard D (1996) Activation of the unliganded estrogen receptor by EGFr involves the MAP kinase pathway and direct phosphorylation. EMBO J 15: 2174–2183.
19. Dutertre M, Smith CL (2003) Ligand-independent interactions of p160/steroid receptor coactivators and CREB-binding protein (CBP) with estrogen receptor-alpha: regulation by phosphorylation sites in the A/B region depends on other estrogen response element gene regulatory machinery. J Biol Chem 278: 29913–29921.
20. Liou YC, Ryo A, Huang HK, Lu PJ, Bronson R, et al. (2002) Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. Proc Natl Acad Sci U S A 99: 1335–1340.
21. Chen L, Kimsey A, Sauter G, Osocodue JM, Lu KP, et al. (2004) Prevalent overexpression of prolyl isomerase Pin1 in human cancers.
22. Khanal P, Namgoong GM, Kang BS, Woo ER, Choi HS (2010) The prolyl isomerase Pin1 enhances HER-2 expression and cellular transformation via its interaction with mitogen-activated protein kinase/ extracellular signal-regulated kinase 1. Mol Cancer Ther 9: 606–616.
23. Rizzolli F, Lucchetti C, Caligiuri I, Marchesi I, Caputo M, et al. (2012) Retinoblastoma tumor-suppressor protein phosphorylation and inactivation depend on direct interaction with Pin1. Cell Death Differ 19: 1152–1161.
24. Rizzolli F, Pramparo T, Sals C, Zuffardi O, De Santis L, et al. (2009) Epigenetic analysis of the critical region I for premature ovarian failure: demonstration of a highly heterochromatic domain on the long arm of the mammalian X chromosome. J Med Genet 46: 505–592.
25. Girardini JE, Polo M, Piazza S, Rustighi A, Marotta C, et al. (2011) A Pin1 mutant p53 axis promotes aggressiveness in breast cancer. Cancer Cell 20: 79–91.
26. Nobili M, Girardini JE, Piazza S, Del Sal G (2011) Wiring the oncogenic circuitry: Pin1 unleashes mutant p53. Oncotarget 2: 654–656.
27. Dutertre M, Smith CL (2003) Ligand-independent interactions of p160/steroid receptor coactivators and CREB-binding protein (CBP) with estrogen receptor-alpha: regulation by phosphorylation sites in the A/B region depends on other estrogen response element gene regulatory machinery. J Biol Chem 278: 29913–29921.
28. Liou YC, Ryo A, Huang HK, Lu PJ, Bronson R, et al. (2002) Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. Proc Natl Acad Sci U S A 99: 1335–1340.
29. Vilgelm A, Lian Z, Wang H, Beauparlant SL, Klein-Szanto A, et al. (2006) Akt-mediated phosphorylation and activation of estrogen receptor-alpha: regulation by phosphorylation sites in the A/B region depends on other receptor domains. Mol Endocrinol 17: 1296–1314.
30. Vilgelm A, Lian Z, Wang H, Beauparlant SL, Klein-Szanto A, et al. (2006) Akt-mediated phosphorylation and activation of estrogen receptor-alpha: regulation by phosphorylation sites in the A/B region depends on other receptor domains. Mol Endocrinol 17: 1296–1314.
31. Rizzolli F, Lucchetti C, Caligiuri I, Marchesi I, Caputo M, et al. (2012) Retinoblastoma tumor-suppressor protein phosphorylation and inactivation depend on direct interaction with Pin1. Cell Death Differ 19: 1152–1161.
32. Rizzolli F, Pramparo T, Sala C, Zuffardi O, De Santis L, et al. (2009) Epigenetic analysis of the critical region I for premature ovarian failure: demonstration of a highly heterochromatic domain on the long arm of the mammalian X chromosome. J Med Genet 46: 505–592.
33. Roberti A, Rizzolli F, Lucchetti C, De Leval I, Giorlando A (2011) Ubiquitin-mediated protein degradation and methylation-induced gene silencing cooperate in the inactivation of the INI1/ARF locus in Burkitt lymphoma cell lines. Cell Cycle 10: 127–134.
34. Liou YC, Ryo A, Huang HK, Lu PJ, Bronson R, et al. (2002) Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. Proc Natl Acad Sci U S A 99: 1335–1340.
35. Berry DA, Girancione C, Henderson IC, Citron ML, Budman DR, et al. (2006) Estrogen-receptor status and outcomes of modern chemotherapy for patients with node-positive breast cancer. JAMA 295: 1638–1647.
36. Ali S, Buluwela L, Coombes RC (2011) Antiestrogens and their therapeutic applications in breast cancer and other diseases. Annual review of medicine 62: 217–232.

37. Allegra JC, Lippman ME, Simon R, Thompson EB, Barbeck A, et al. (1978) Association between steroid hormone receptor status and disease-free interval in breast cancer. Cancer Treat Rep 63: 1271–1277.

38. Osborne CK, Yochimowitiz MG, Knight 3rd WA, McGuire WL (1980) The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer 46: 2884–2890.

39. Williams MR, Todd JH, Ellis IO, Dowle CS, Haybittle JL, et al. (1987) Oestrogen receptors in primary and advanced breast cancer: an eight year review of 704 cases. Br J Cancer 55: 67–73.

40. Gray (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 365: 1687–1717.

41. Lee KY, Lee JW, Nam HJ, Shim JH, Song Y, et al. (2011) PI3-kinase/p38 kinase-dependent E2F1 activation is critical for Pin1 induction in tamoxifen-resistant breast cancer cells. Mol Cells 32: 107–111.

42. Valley CC, Metivier R, Solodin NM, Fowler AM, Maneck MT, et al. (2005) Differential regulation of estrogen-inducible proteolysis and transcription by the estrogen receptor alpha N terminus. Mol Cell Biol 25: 5417–5428.

43. Sentis S, Le Romancer M, Bianchin C, Rostan M-C, Corbo L (2005) Sumoylation of the estrogen receptor alpha hinge region regulates its transcriptional activity. Molecular endocrinology (Baltimore, Md) 19: 2671–2684.

44. Skliris GP, Nugent ZJ, Rowan BG, Penner CR, Watson PH, et al. (2010) A phosphorylation code for oestrogen receptor-alpha predicts clinical outcome to endocrine therapy in breast cancer. Endocr Relat Cancer 17: 589–597.

45. Rizzolio F, Caligiuri I, Lacchetti C, Fratamico R, Tomei V, et al. (2012) Dissecting Pin1 and phospho-pRb regulation. J Cell Physiol.

46. Yamashita H, Nishio M, Toyama T, Sugiuara H, Kondo N, et al. (2008) Low phosphorylation of estrogen receptor alpha (ERalpha) serine 118 and high phosphorylation of ERalpha serine 167 improve survival in ER-positive breast cancer. Endocr Relat Cancer 15: 755–763.

47. Jiang J, Sarwar N, Peston D, Kalliskaya E, Shousha S, et al. (2007) Phosphorylation of estrogen receptor-alpha at Ser167 is indicative of longer disease-free and overall survival in breast cancer patients. Clin Cancer Res 13: 5769–5776.

48. Sarwar N, Kim JS, Jiang J, Peston D, Srinath HD, et al. (2006) Phosphorylation of ERalpha at serine 118 in primary breast cancer and in tamoxifen-resistant tumours is indicative of a complex role for ERalpha phosphorylation in breast cancer progression. Endocr Relat Cancer 13: 851–861.

49. Trowbridge JM, Rogatsky I, Garabedian MJ (1997) Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex. Proceedings of the National Academy of Sciences of the United States of America 94: 10132–10137.

50. Henry MK, Nimbalkar D, Hohl RJ, Quelle FW (2004) Cytokine-induced phosphoinositide 3-kinase activity promotes Cdk2 activation in factor-dependent hematopoietic cells. Exp Cell Res 299: 257–266.

51. Wierod L, Rosseland CM, Lindeman B, Okvold MP, Grosvik H, et al. (2007) CDK2 regulation through PI3K and CDK4 is necessary for cell cycle progression of primary rat hepatocytes. Cell Prolif 40: 475–487.

52. Takeda A, Osaki M, Adachi K, Honjo S, Ito H (2004) Role of the phosphatidylinositol 3′-kinase-Akt signal pathway in the proliferation of human pancreatic ductal carcinoma cell lines. Pancreas 28: 353–358.

53. Lao Y, Wei Y, Zhou X, Yang JY, Dai C, et al. (2009) Peptidyl-prolyl cis/trans isomerase Pin1 is critical for the regulation of PI3K/Akt stability and activation phosphorylation. Oncogene 28: 2436–2445.