Prolyl isomerase Pin1: a catalyst for oncogenesis and a potential therapeutic target in cancer

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Summary

Phosphorylation of proteins on serine or threonine residues preceding proline (Ser/Thr-Pro) is a major intracellular signaling mechanism. The phosphorylated Ser/Thr-Pro motifs in a certain subset of phosphoproteins are isomerized specifically by the peptidyl-prolyl cis-trans isomerase Pin1. This post-phosphorylation isomerization can lead to conformational changes in the substrate proteins and modulate their functions. Pin1 interacts with a number of mitotic phosphoproteins, and plays a critical role in mitotic regulation. Recent work indicates that Pin1 is overexpressed in many human cancers and plays an important role in oncogenesis. Pin1 regulates the expression of cyclin D1 by cooperating with Ras signaling and inhibiting the interaction of β-catenin with the tumor suppressor APC and also directly stabilizing cyclin D1 protein. Furthermore, *PIN1* is an E2F target gene essential for the Neu/Ras-induced transformation of mammary epithelial cells. Pin1 is also a critical regulator of the tumor suppressor p53 during DNA damage response. Given its role in cell growth control and oncogenesis, Pin1 could represent a new anti-cancer target.

Key words: Phosphorylation, Prolyl-isomerase, Pin1, Post-phosphorylation regulation, Signal transduction, Oncogenesis

Introduction

The phosphorylation of proteins on serine or threonine residues that immediately precede proline residues (Ser/Thr-Pro) is an important signaling mechanism controlling many cellular processes, such as cell cycle regulation, transcription, cell differentiation and proliferation (Blume-Jensen and Hunter, 2001; Hunter, 1995; Hunter, 1998; Lu, K. P. et al., 2002b). The deregulation of this elaborate signaling mechanism can result in cell transformation and oncogenesis. Therefore, precise pre- and post-phosphorylation regulatory mechanisms have evolved that maintain steady-state intracellular signaling.

Ser/Thr–Pro motifs are the major phosphorylation sites for a large superfamily of 'proline-directed' kinases, including cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs) and glycogen synthase kinase 3β (GSK-3β), and conversely they are dephosphorylated by Ser/Thr phosphatases, including PP2A, FCP1 and calcineurin (Lu, K. P. et al., 2002b). Furthermore, MAPK ERK2 and CDK2, as well as the Ser/Thr phosphatase PP2A, are conformation specific, preferentially phosphorylating/dephosphorylating the trans isomer (Brown et al., 1999; Weiwad et al., 2000; Zhou et al., 2000). Ser/Thr phosphorylation has for a long time been believed to regulate the function of proteins by altering their conformations; however, little is known about the actual conformational changes and their importance. The identification and characterization of a peptidyl-prolyl cis/trans isomerase (PPIase), Pin1, has led to the discovery of a novel post-phosphorylation regulatory mechanism (Lu et al., 1996; Ranganathan et al., 1997; Yaffe et al., 1997). Pin1 binds to and isomerizes the peptidyl-prolyl bond in specific phosphorylated Ser/Thr-Pro motifs and thereby induces conformational changes in its target proteins (Albert et al., 1999; Arevalo-Rodriguez et al., 2000; Hsu et al., 2001; Kops et al., 2002; Liou et al., 2002; Lu et al., 1999a; Lu et al., 1999b; Ryo et al., 2002; Ryo et al., 2001; Shen et al., 1998; Stukenberg and Kirschner, 2001; Wu et al., 2000; Wulf et al., 2002; Wulf et al., 2001; Yaffe et al., 1997; Zacchi et al., 2002; Zheng et al., 2002; Zhou et al., 2000). These conformational changes can have profound effects on the function of Pin1 substrates, modulating their activity, phosphorylation status, protein-protein interactions, subcellular localization and stability (Fig. 1). For example, Pin1 can bind to and induce conformational changes in the mitotic phosphatase Cdc25C and the microtubule-binding protein tau, after they have been phosphorylated on specific Ser/Thr-Pro motifs. Such conformational changes can directly inhibit the ability of phosphorylated Cdc25C to dephosphorylate and activate Cdc2 (Shen et al., 1998; Stukenberg and Kirschner, 2001; Zhou et al., 2000), or restore the ability of phosphorylated tau to promote microtubule assembly (Lu et al., 1999a). Furthermore, such conformational changes can also regulate the dephosphorylation of Cdc25C and tau because phosphatases such as PP2A dephosphorylate only the trans isoform of phosphorylated Ser/Thr-Pro motifs (Zhou et al., 2000). Thus, phosphorylation-dependent prolyl isomerization is a new post-phosphorylation signaling mechanism.

Pin1 was originally identified in a yeast two-hybrid screen as a human protein that interacts physically and functionally with a mitotic kinase and was the first peptidyl-prolyl cis/trans isomerase (PPIase) shown to be essential for cell division in yeast and human cells (Lu et al., 1996). Pin1 homologues are

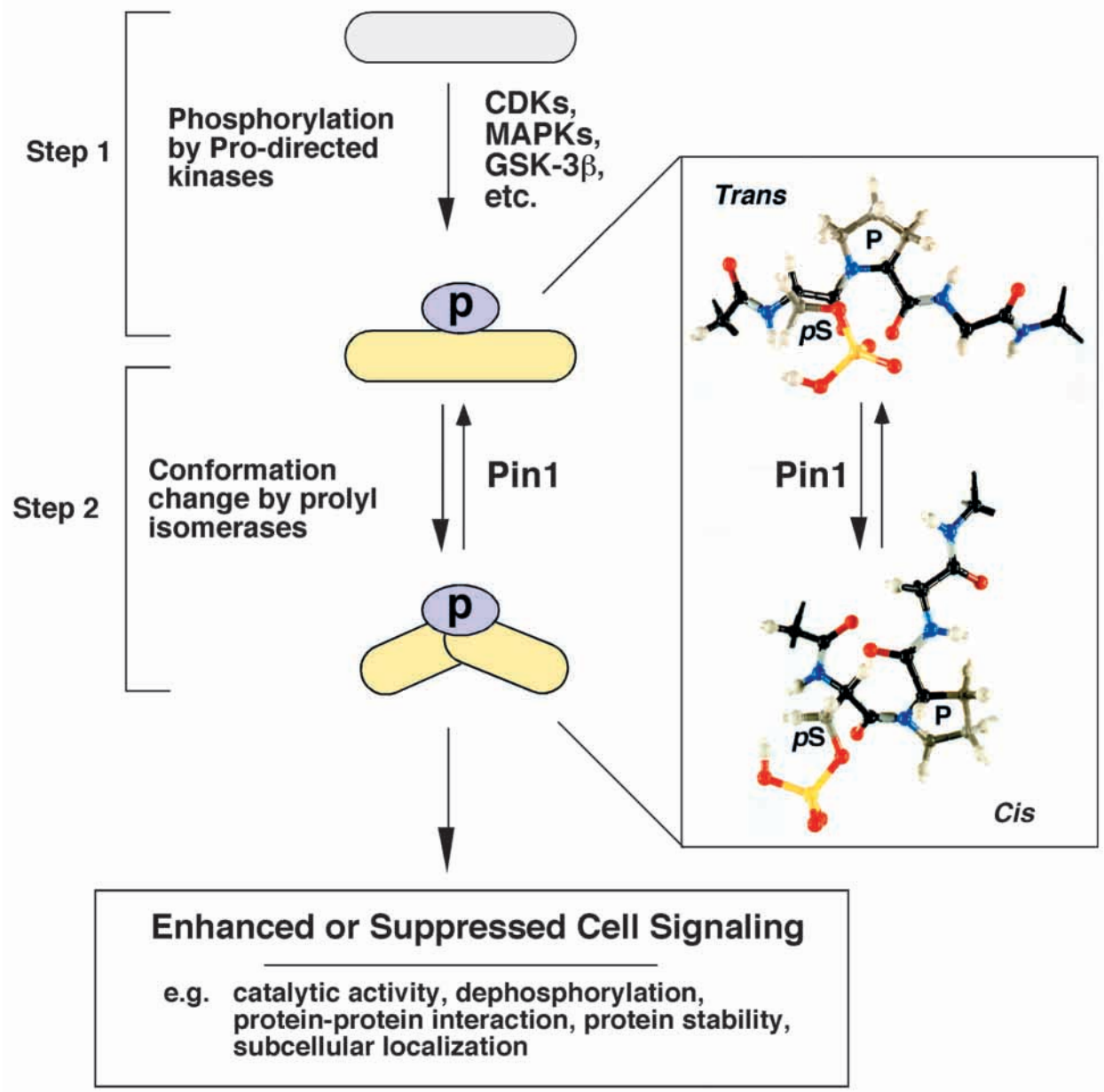

Fig. 1. A novel post-phosphorylation regulatory mechanism in phosphorylation signaling. Phosphorylation of proteins by proline-directed kinases (e.g. CDKs, MAPKs, GSK-3β) creates binding sites for the prolyl-isomerase Pin1 (1st step). Subsequent prolyl-isomerization by Pin1 induces conformational changes and thereby regulates the function of target proteins (2nd step).

highly conserved in eukaryotes (Huang et al., 2001; Landrieu et al., 2000; Metzner et al., 2001; Yao et al., 2001; Zhou et al., 1999), and the budding yeast homologue, Ess1p/Ptrf1p, was isolated a long time ago but did not have any previously known activity (Hanes et al., 1989; Hani et al., 1995). With the exception of the plant enzymes, which appear to contain only PPIase domains, most other Pin1-type PPIases also contain an N-terminal WW domain. The function of the WW domain is to target the enzyme to its substrates, where the PPIase domain is both sufficient and necessary to catalyze the conformational change and to carry out the essential function of this enzyme. Depletion of Pin1 causes mitotic arrest and apoptosis in budding yeast and tumor cell lines (Lu et al., 1996), and Pin1 is required for the DNA replication checkpoint and G2/M transition in *Xenopus* extracts (Winkler et al., 2000). Pin1 has been shown to be involved in the regulation of many other cellular events, such as cell cycle progression, transcriptional regulation and cell proliferation and differentiation (Albert et al., 1999; Arevalo-Rodriguez et al., 2000; Crenshaw et al., 1998; Gerez et al., 2000; Hani et al., 1999; Hsu et al., 2001; Kamimoto et al., 2001; Lavoie et al., 2001; Liou et al., 2002; Liu et al., 2001; Messenger et al., 2002; Morris et al., 1999; Pathan et al., 2001; Patra et al., 1999; Rippmann et al., 2000; Ryo et al., 2002; Ryo et al., 2001; Shen et al., 1998; Wu et al., 2000; Wulf et al., 2001). Furthermore, it is involved in the DNA damage response, regulating p53 function (Wulf et al., 2002; Zacchi et al., 2002; Zheng et al., 2002). Moreover, it is also involved in Alzheimer's disease (Lu et al., 1999a; Zhou et al.,

Table 1. Representative known Pin1 substrates

Protein	Function	References
NIMA	Mitotic kinase	Lu et al., 1996
Cdc25C	Protein phosphatase for Cdc2	Yaffe et al., 1997; Crenshaw et al., 1998; Shen et al., 1998; Lu et al., 1999b; Zhou et al., 2000; Stukenberg and Kirschner, 2001
Plk 1	Mitotic kinase	Yaffe et al., 1997; Crenshaw et al., 1998; Shen et al., 1998
Cdc27	Anaphase-promoting complex component	Yaffe et al., 1997; Shen et al., 1998
Rab4	GTP-binding protein	Yaffe et al., 1997; Gerez et al., 2000
p70/S6 kinase	Protein kinase	Yaffe et al., 1997
Wee1	Mitotic kinase	Shen et al., 1998
Myt1	Mitotic kinase	Shen et al., 1998; Wells et al., 1999
CENP-F	Kinetokore protein	Shen et al., 1998
Incenp	Inner centromere protein	Shen et al., 1998
Tau	Microtubule-interacting protein	Lu et al., 1999a; Zhou et al., 2000
Pol II	RNA polymerase II	Albert et al., 1999; Morris et al., 1999; Verdecia et al., 2000; Wu et al., 2000; Kops et al., 2002
Sin3–Rpd3	Histone deacetylase	Arevalo-Rodriguez et al., 2000
NHERF-1	Na+/H+ exchanger regulatory factor 1	He et al., 2001
KRMP1	Kinesin-related protein	Kamimoto et al., 2001
hSpt5	A DRB sensitivity-inducing factor component	Lavoie et al., 2001
Bcl-2	Anti-apoptotic factor	Pathan et al., 2001; Basu et al., 2002
c-Jun	Transcription factor	Wulf et al., 2001
β-catenin	Transcription activator	Ryo et al., 2001
NFAT	Transcription factor	Liu et al., 2001
Cf-2	Transcriptional repressor	Hsu et al., 2001
Cyclin D1	Cell cycle regulator	Liou et al., 2002; Ryo et al., 2002
CK2	Protein kinase	Messenger et al., 2002
p53	Transcription factor	Wulf et al., 2002; Zacchi et al., 2002; Zheng et al., 2002

2000) and cancer (Liou et al., 2002; Ryo et al., 2002; Ryo et al., 2001; Wulf et al., 2001).

Here we review recent studies demonstrating the role of Pin1 in cell growth control and oncogenesis and discuss the feasibility of Pin1 as a potential therapeutic target for anti-cancer treatment. Comprehensive recent reviews on function and regulation of Pin1 (Lu, K. P. et al., 2002b; Zhou et al., 1999), as well as its specific role in transcription (Shaw, 2002) and in Alzheimer's disease (Lu, K. P. et al., 2002a), are available elsewhere.

Regulation of cyclin D1 expression

At least 20 proteins have been shown to be Pin1 targets (Table 1). Of these known Pin1 substrates, cyclin D1 is the most extensively studied both in vitro and in vivo (Liou et al., 2002; Ryo et al., 2002; Ryo et al., 2001; Wulf et al., 2001). The connection between Pin1 and cyclin D1 was originally identified in screens for Pin1 expression in human breast cancer tissues (Wulf et al., 2001). Indeed, Pin1 is overexpressed in several human cancers, including breast, lung and prostate cancers and its expression levels positively correlate with the tumor grade in breast cancer (Ryo et al., 2001; Wulf et al., 2001). In addition, Pin1 is overexpressed in all breast cancer cell lines examined when compared with non-transformed or primary mammary epithelial cells (Wulf et al., 2001), and Pin1 is one of the genes suppressed by overexpression of wild-type BRCA1 (MacLachlan et al., 2000).

Elevated Pin1 levels in breast cancer significantly correlate with cyclin D1 overexpression (Ryo et al., 2002; Ryo et al., 2001; Wulf et al., 2001). In fact, we have found that Pin1 levels in cyclin D1 overexpressing tumors are on average about twice as high as those in cyclin-D1-negative tumors. This is significant given that cyclin D1 is an essential downstream target for mammary tumorigenesis. Cyclin D1 is overexpressed in about half of breast cancer patients (Bartkova et al., 1994; Gillett et al., 1994). Overexpression of cyclin D1 contributes to cell transformation (Alt et al., 2000; Hinds et al., 1994), inhibition of cyclin D1 expression by antisense expression causes growth arrest in tumor (Arber et al., 1997; Driscoll et al., 1997; Kornmann et al., 1998; Schrump et al., 1996) and disruption of the cyclin D1 gene in mice completely suppresses the ability of Ha-Ras or Her2/Neu to induce tumor development in mammary glands (Yu et al., 2001). In breast cancer tissues, Her2/Neu overexpression correlates with Pin1 overexpression, although this correlation did not reach statistical significance, probably because of the small number of Her2/Neu-positive patients in the study (Wulf et al., 2001). It is of interest, though, that Pin1 levels were 1.7-2 times higher in patients who are either Her2/Neu positive, or negative for estrogen receptor expression (Wulf et al., 2001). Further studies in larger cohorts may clarify the relationship between Pin1 expression and these unfavorable biochemical markers, and establish whether Pin1 expression would be a useful additional marker for breast cancer prognosis.

Cooperation with the Ras/AP-1 signaling pathway

The AP-1 complex, which includes transcription factors such as Jun, Fos and FosB, regulates a wide range of cellular processes, including cell proliferation, cell death, survival and differentiation, and has a binding site in the cyclin D1 promoter (Shaulian and Karin, 2002). The significance of Jun activation for cell proliferation and cyclin D1 induction has been studied in mice lacking AP-1 components. *Fos*/*FosB* double knockout and *Jun* knockout mouse embryonic fibroblasts (MEFs) have reduced cyclin D1 levels and display severe proliferative defects (Behrens et al., 1999; Brown et al., 1998; Shaulian and Karin, 2001; Shaulian and Karin, 2002). Ras signaling

activates the proline-directed MAP kinases p38 and JNK/SAPK, which phosphorylate Jun on two critical N-terminal Ser-Pro motifs at Ser63 and Ser73, thereby enhancing its transcriptional activity (Albanese et al., 1999; Albanese et al., 1995; Bakiri et al., 2000; Binetruy et al., 1991; Derijard et al., 1994; Shaulian and Karin, 2001; Whitmarsh and Davis, 1996). How the activity of Jun is further regulated after phosphorylation has not been known until recently.

We have found that Pin1 not only binds phosphorylated Jun but also increases its ability to activate the cyclin D1 promoter in cooperation either with activated JNK or oncogenic Ha-Ras. In contrast, inhibition of endogenous Pin1 reduces the transcriptional activity of phosphorylated Jun, indicating that endogenous Pin1 is also required for optimal activation. Thus, Pin1 is a potent modulator of phosphorylated Jun in inducing cyclin D1 expression, presumably by regulating the conformation of the phosphorylated Ser-Pro motifs in Jun (Wulf et al., 2001). Jun is basically a positive regulator of cell proliferation (Behrens et al., 1999; Brown et al., 1998; Shaulian and Karin, 2001; Shaulian and Karin, 2002), and the Pin1-induced conformational changes in Jun potentially affect its ability to form homo- or hetero-dimers and/or its DNA binding activity. However, further studies are necessary to define the molecular mechanisms by which Pin1 affects Jun function.

Activation of the Wnt/β-catenin pathway

Wnt/β-catenin signaling is involved in control of gene expression, cell adhesion and cell polarity (Kinzler and Vogelstein, 1996; Moon et al., 2002; Morin, 1999; Polakis, 2000). Activation of the Wnt/β-catenin signaling pathway is a major feature of human cancers (Kinzler and Vogelstein, 1996; Polakis, 2000). Deregulation of this signaling pathway has been found in a subset of human malignancies that carry mutations in proteins participating in this pathway, such as APC (adenomatous polyposis coli), axin and β-catenin itself (Kinzler and Vogelstein, 1996; Polakis, 2000; Satoh et al., 2000). The end result of this aberrant activation is always the cytosolic stabilization of β-catenin, which enhances the transcription of a number of target genes, including the cyclin D1 gene and *Myc*, which can lead to oncogenesis (Behrens et al., 1996; He et al., 1998; Mann et al., 1999; Molenaar et al., 1996; Tetsu and McCormick, 1999). Mutations in APC or β-catenin are often found in certain tumor types, such as colon cancer (Kinzler and Vogelstein, 1996; Morin, 1999; Polakis, 2000), but they are rarely observed in others, such as breast cancer (Jonsson et al., 2000; Lin et al., 2000; Schlosshauer et al., 2000). However, there is compelling evidence for a crucial role for β-catenin signaling in the tumorigenesis of breast cancer (Jonsson et al., 2000; Lin et al., 2000; Roose et al., 1999; Schlosshauer et al., 2000). Furthermore, β-catenin levels are significantly upregulated and are a strong and independent prognostic factor in human breast cancer patients (Lin et al., 2000).

A differential display screen for genes regulated by Pin1 that compared inducible Pin1-overexpressing breast cancer MCF-7 cells and control cells identified 17 known genes (Ryo et al., 2001). Interestingly, four of the 12 genes whose expression is upregulated are targets of β-catenin and its transcriptional partner TCF: those encoding cyclin D1, Myc, PPAR-delta and fibronectin (Gradl et al., 1999; He et al., 1999; He et al., 1998; Tetsu and McCormick, 1999). Overexpression or depletion of Pin1 in cell lines has been shown to regulate the stability and subcellular localization of β-catenin (Ryo et al., 2001). In addition, Pin1 can activate the cyclin D1 promoter not only through AP-1 sites but also through TCF-binding sites that are present (Ryo et al., 2001). Upregulation of Pin1 in breast cancer strongly correlates with increased β-catenin levels in the tumors examined, whereas β-catenin levels are decreased in Pin1-knockout mouse tissues (Ryo et al., 2001).

How does Pin1 regulate β-catenin levels? As shown above, conformational changes caused by Pin1-catalyzed prolyl-isomerization can affect protein stability, phosphorylation status and protein-protein interactions (Hsu et al., 2001; Kops et al., 2002; Liou et al., 2002; Lu et al., 1999a; Lu et al., 1999b; Ryo et al., 2002; Ryo et al., 2001; Shen et al., 1998; Stukenberg and Kirschner, 2001; Wulf et al., 2002; Wulf et al., 2001; Yaffe et al., 1997; Zacchi et al., 2002; Zheng et al., 2002; Zhou et al., 2000). Pin1 binds exclusively to phosphorylated β-catenin, and its binding site has been mapped to the $pSer^{246}$-Pro motif (Ryo et al., 2001). This motif is located at an exposed loop region between the two helixes at the third armadillo repeat interface, and it is next to the surface that interacts with APC in the three-dimensional structure of β-catenin (Graham et al., 2000; Huber et al., 1997; von Kries et al., 2000). APC is the shuttling protein that exports nuclear β-catenin to the cytoplasm for degradation (Bienz, 2002; Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). Mutations of Phe^{253} and Phe^{293} in β-catenin abolish its ability to bind to APC (Graham et al., 2000; Huber et al., 1997; von Kries et al., 2000). Similarly, Pin1 binding and isomerization specifically inhibits the interaction between β-catenin and APC, resulting in the nuclear accumulation and stabilization of β-catenin. Pin1-dependent prolyl-isomerization thus appears to be a novel mechanism for the regulation of β-catenin–APC interaction. Given the overexpression of Pin1 in many cancers, this mechanism might up-regulate β-catenin activity in tumors such as breast cancer, in which APC and/or β-catenin mutations are not common (Ryo et al., 2001).

Regulation of cyclin D1 protein levels

Pin1-knockout mice were originally reported to develop normally and to have no phenotype (Fujimori et al., 1999). More recent analyses indicate that these mice display a range of cell proliferative abnormalities, including decreased body size, testicular atrophy and retinal degeneration, a phenotype strongly reminiscent of the cyclin-D1-knockout mice (Liou et al., 2002). Most strikingly, the breast epithelial compartment in *Pin1*-null mice cannot undergo the massive proliferative changes associated with pregnancy. Importantly, cyclin D1 protein levels are significantly decreased in every tissue that displays a severe phenotype (Liou et al., 2002). These results strongly suggest that Pin1 is essential for the regulation of cyclin D1 in vivo. Interestingly, mouse models in which AP-1 or β-catenin/APC function is perturbed do not display defects associated with lack of cyclin D1 (Behrens et al., 1999; Brown et al., 1998; Haegel et al., 1995; Shaulian and Karin, 2001), indicating that other Pin1-dependent mechanisms for regulating cyclin D1 exist. Indeed, cyclin D1 mRNA levels in *Pin1*-knockout MEFs are significantly reduced probably

because of defective Jun/AP-1 and β-catenin/TCF pathways. However, the reduction of cyclin D1 protein levels is disproportionally greater (Liou et al., 2002). Phosphorylation of cyclin D1 by GSK-3β on Thr286-Pro site regulates turnover and localization of cyclin D1 by enhancing its binding to CRM1, a nuclear exporter of cyclin D1, which leads to degradation of cyclin D1 in the cytoplasm (Alt et al., 2000; Diehl et al., 1998; Diehl et al., 1997; Fukuda et al., 1997). Pin1 can bind to and presumably isomerize the phosphorylated Thr286-Pro motif in cyclin D1, which may inhibit its interaction with CRM1. This would stabilize cyclin D1 by preventing its nuclear export and proteolysis in the cytoplasm (Liou et al., 2002). Pin1 thus seems to regulate cyclin D1 function at both the transcriptional and post-translational levels, and this may explain why the *Pin1*-null phenotype resembles the cyclin-D1-null phenotype. It is also consistent with the finding that Pin1-deficient MEFs cannot effectively restart proliferation in response to serum stimulation after G0 arrest (Fujimori et al., 1999). These results indicate that Pin1 is required for progression through G0 to S in addition to mitosis.

Regulation of pin1 transcription and function by oncogenic pathways

How is Pin1 activated in cancer cells? The *PIN1* promoter sequence has neither a TATA nor CAAT box but has two putative GC boxes and three putative E2F-binding sites (Ryo et al., 2002). Indeed, E2F family proteins activate the *PIN1* promoter through these E2F-binding sites. E2F proteins also bind the *PIN1* promoter in vitro and in vivo, and increased Pin1 levels in breast cancer cell lines correlate with an increase in binding of E2F to the *PIN1* promoter. Moreover, overexpression of E2F enhances *PIN1* promoter activity and mRNA levels in breast cancer cells. In common with many other E2F-target genes (Fry et al., 1997; Nevins, 2001; Ohtani et al., 1995), *PIN1* transcription and its protein levels fluctuate during cell cycle progression in non-neoplastic cells (Ryo et al., 2002) but not in transformed cells (Shen et al., 1998). Interestingly, aberrantly high E2F1 levels have been described in breast cancer (Zhang et al., 2000), and therefore it is possible that deregulation of E2F plays a key role in the upregulation of Pin1 in breast cancer. Since deregulation of the Rb/E2F pathway is also found in many other cancer types and contributes to the oncogenesis of a number of human cancers (Johnson and Schneider-Broussard, 1998; Nevins, 2001; Weinberg, 1995), deregulation of the Rb/E2F pathway may cause Pin1 overexpression in other cancer cells.

In addition to being transcriptionally regulated, Pin1 is also regulated by post-translational controls. One such regulatory mechanism is phosphorylation. Phosphorylation of the Pin1 WW domain inhibits its ability to bind target proteins and regulates the subcellular localization of Pin1 (Lu, P. J. et al., 2002). Dephosphorylated Pin1 accumulates during the G2/M transition in HeLa cells, whereas in G1 and S phase phosphorylated Pin1 is predominant (Lu, P. J. et al., 2002). In human breast tumors, the dephosphorylated, and presumably active, form of Pin1 accumulates (Wulf et al., 2001). It will be important to identify the specific kinase responsible because, theoretically, this kinase would be able to inhibit Pin1 function, thereby suppressing its ability to activate oncogenic pathways as described above. Phosphospecific antibodies will help us to assess more accurately the ratio of phosphorylated to dephosphorylated Pin1 and may become an important tool for identifying the kinase/phosphatase activities regulating the phosphorylation status of Pin1. Finally, Pin1 levels have been shown to be decreased upon prolonged exposure to the microtubule-targeting drug Taxol, which can apparently be prevented by some proteasome inhibitors; this suggests that Pin1 is also subjected to proteolytic regulation (Basu et al., 2002). However, direct evidence for such a regulatory mechanism has not yet been provided.

Pin1 overexpression and cell transformation

Recent data support the notion that Pin1 can at least partially transform mammary epithelial cells (Ryo et al., 2002). Pin1 confers anchorage-independent cell growth on the non-transformed mammary epithelial cell line MCF-10A (Ryo et al., 2002). Furthermore, its overexpression interferes with normal cell differentiation and acinal formation in a three-dimensional matrigel assay (Muthuswamy et al., 2001; Petersen et al., 1992; Ryo et al., 2002). However, Pin1 overexpression does not affect cell growth or cell morphology under normal culture conditions. Overexpression of Pin1 might thus trigger some early events during cell transformation (Ryo et al., 2002), although it remains to be determined whether Pin1 itself is sufficient for transformation in vivo. It is possible that elevated Pin1 levels become oncogenic only after a ‘first hit’, i.e. activation of an oncogenic pathway that leads to substrate phosphorylation that allows Pin1 to exert this function. The observation that Pin1 greatly enhances and facilitates transformation by oncogenic Neu and Ras in mammary epithelial cells is consistent with this hypothesis (Ryo et al., 2002).

Neu or Ras signaling is frequently deregulated in breast cancers, although mutations and amplifications of these genes are rarely observed (Andrechek and Muller, 2000; Harari and Yarden, 2000). Transgenic overexpression of MMTV-Ha-Ras or MMTV-Neu potently induces mammary tumors by stimulating cyclin D1. However, transgenic overexpression of MMTV-cyclin D1 is much less tumorigenic (Muller et al., 1988; Sinn et al., 1987; Wang et al., 1994). In addition, constitutive overexpression of cyclin D1 alone cannot transform MCF-10A cells, nor is it sufficient to prevent G1 arrest induced by EGF deprivation (Chou et al., 1999). These discrepancies could be explained by the findings that cyclin D1 is regulated not only by transcriptional activation but also by the post-translational stabilization described above. In contrast to wild-type cyclin D1, the mutant cyclin $D1^{T286A}$ is stable and functions as a constitutively active mutant that can potently transform fibroblasts (Alt et al., 2000). Both transcriptional activation and post-translational stabilization of cyclin D1 thus seem to be critical for tumor development induced by Neu/Ras signaling.

Similarly to cyclin D1, Pin1 is highly overexpressed in the mammary glands of transgenic mice that overexpress MMTV-Neu or MMTV-Ha-Ras (Muller et al., 1988; Ryo et al., 2002; Sinn et al., 1987). Inhibition of Pin1 by a dominant negative mutant or an antisense construct dramatically reduces both cell proliferation and the transformation induced by the Neu and Ras oncogenes. This reduction can be reversed by expression

of the constitutively active cyclin D1 T286A mutant that is resistant to Pin1 inhibition (Ryo et al., 2002). These results suggest that cyclin D1 is a specific downstream target of Pin1 for oncogenesis. Cyclin D1 is overexpressed in 50% of all breast cancers, but genetic amplification accounts for only 10% of this overexpression (Sutherland and Musgrove, 2002). Pin1 therefore probably plays an important role in maintaining cyclin D1 levels sufficient for transformation of mammary epithelial cells.

Regulation of p53 in the DNA damage response

The tumor suppressor protein p53 regulates multiple cellular functions, including cell cycle checkpoints, genomic stability and apoptosis (Colman et al., 2000; Lakin and Jackson, 1999; Meek, 1999; Ryan et al., 2001; Taylor and Stark, 2001; Wahl and Carr, 2001). DNA damage leads to the stabilization and accumulation of p53, which plays a pivotal role in transcriptional activation of the cell cycle inhibitor p21 and cell cycle arrest. This prolonged half-life of p53 is at least partially due to its dissociation from the ubiquitin ligase MDM2. The increase in p53 stability and/or transcriptional activity depends critically on its phosphorylation on multiple serine/threonine residues, including those preceding prolines (Abraham et al., 2000; Blaydes et al., 2001; Bulavin et al., 1999; Buschmann et al., 2001; Milner et al., 1990; Sakaguchi et al., 1998; Sanchez-Prieto et al., 2000; Turenne et al., 2001). Recent results indicate a new role of Pin1 in the DNA damage response. Pin1 is an indispensable positive regulator of p53 in response to DNA damage induced by genotoxic drug treatment or UV radiation (Zacchi et al., 2002; Zheng et al., 2002) or ionizing radiation (Wulf et al., 2002; Zacchi, 2002). DNA damage enhances the specific interaction between Pin1 and p53, which depends on the WW domain in Pin1 and specific phosphorylated Ser-Pro motifs in p53. Pin1 binds to p53 at phosphorylated Ser33 and Ser46 following exposure to ionizing radiation (Wulf et al., 2002) and at phosphorylated Ser33, Thr81 and Ser315 following exposure to UV radiation or genotoxic drug treatment (Zacchi et al., 2002; Zheng et al., 2002). More importantly, Pin1 is required for the stabilization of the p53 protein after DNA damage, preventing p53 from binding to its ubiquitin ligase, MDM2 (Wulf et al., 2002; Zacchi et al., 2002). In addition, Pin1 increases the transcriptional activity of p53 towards the *p21* and *MDM2* promoters (Wulf et al., 2002; Zacchi et al., 2002; Zheng et al., 2002). Given that phosphorylation of p53 by various kinases, such as Chk1, ATM and MAP kinases, is believed to play a central role in induction of p53 by DNA damage, these results indicate that Pin1-dependent phosphorylation-dependent isomerization is a new and critical mechanism to control p53 stability and function after it has been phoshorylated.

The physiological consequences of Pin1 loss in normal cells are still controversial. Zheng et al. (Zheng et al., 2002) and Wulf et al. (Wulf et al., 2002) show that Pin1 is required for maintaining the DNA-damage cell cycle checkpoint by inducing the cell cycle inhibitor p21, which will protect cells from DNA damage-induced cell death (Wulf et al., 2002; Zheng et al., 2002). In contrast, Zacchi et al. claim that Pin1 accelerates apoptosis by enhancing pro-apoptotic genes downstream of p53, such as Bax and DR2/Killer (Zacchi et al., 2002). This discrepancy may reflect the fact that p53 induces sets of genes required for cell cycle arrest and apoptosis, depending on the cellular context and intensity and timing of the respective DNA damage (Colman et al., 2000; Lakin and Jackson, 1999; Meek, 1999; Ryan et al., 2001; Taylor and Stark, 2001; Wahl and Carr, 2001). Careful in vivo analysis of the effects of different types of genotoxic insult may clarify whether Pin1-mediated prolyl isomerization of p53 directs the cells in a given cell towards apoptosis or towards cell cycle arrest.

These results are initially counterintuitive: why would a protein that amplifies oncogenic signals also activate a tumor suppressor gene? Proline-directed phosphorylation plays an important role in both the promotion and suppression of oncogenesis, and therefore Pin1 is likely to be involved in both processes. It has been well established that many proteins are involved in both processes. For example, transcription factors such as Myc and E2F family members participate in a complex signaling network that regulates cell growth, differentiation, cell survival and apoptosis in non-malignant cells. Only in a permissive environment will overexpressed or mutated forms of these proteins contribute to carcinogenesis (Oster et al., 2002; Trimarchi and Lees, 2002; Zhou and Hurlin, 2001). Therefore, it may be important to distinguish the physiological function of Pin1 in normal cells from its pathological role in cancers where Pin1 is deregulated. It is likely that, under physiological conditions in normal cells, Pin1-mediated p53 regulation is important for cell cycle checkpoint regulation and the maintenance of genomic stability. In cancer cells, however, this mechanism may be defective because oncogenic signalling pathways induced by Pin1 overexpression may override the DNA damage repair mechanisms and/or because p53 is absent or mutated in many cancer cell types. Interestingly, Pin1 can also stabilize p53 mutants with the same efficacy as the wild-type protein (G.W. and K.P.L., unpublished results). Since a cellular environment in which Myc and/or Ras expression is deregulated can favor the selection of p53 mutations (Chikatsu et al., 2002) and since some p53 mutants function as dominant negative mutants (de Vries et al., 2002; Monti et al., 2002), Pin1 overexpression in the context of a mutated *p53* gene might even contribute to genomic instability in cancer cells. However, further studies are needed to define the physiological and pathological roles of Pin1-mediated p53 regulation.

Is Pin1 an oncogene or a catalyst for oncogenic activation?

A defining feature of oncogenes is that their mutation or amplification is associated with oncogenesis. To date, genetic alterations of the *PIN1* gene in cancer cells have not yet been described. However, Pin1 levels are upregulated in cancer cells, probably as a result of the deregulation of the E2F/Rb pathway, which occurs in >80% of human malignancies (Nevins, 2001). The activity of Pin1 depends critically on the phosphorylation status of its substrate proteins (Liou et al., 2002; Lu et al., 1999b; Ryo et al., 2001; Shen et al., 1998; Stukenberg and Kirschner, 2001; Wulf et al., 2001; Yaffe et al., 1997; Zhou et al., 2000). Without prior phosphorylation of these targets on Ser/Thr-Pro motifs, Pin1 cannot bind and catalyze prolyl-isomerization. In cancer cells, a wave of serine and threonine phosphorylation occurs as a result of oncogenic signaling (Blume-Jensen and Hunter, 2001; Hunter, 1995). We propose

that Pin1 overexpression cooperates with these activated kinases to promote cell proliferation and transformation. In this model, Pin1 would respond to and translate oncogenic signaling into the actual events of cancer cell growth. Whereas Pin1 itself may not be sufficient for complete cell transformation, it would be an indispensable translator and amplifier of oncogenic signal transduction. The fact that Pin1 depends on the presence of oncogenes such as Ras or Neu to transform cells fully in vitro is consistent with this idea. This means that the action of Pin1 might depend entirely on the cellular context and may vary significantly with cell type, proliferative status and age. For example, *Pin1*-knockout mice develop normally and do not show any significant phenotype at a young age. However, after several months, the mice exhibit age-dependent proliferative disorders in specific tissues (Liou et al., 2002). Studies on the incidence and distribution of tumor development in *Pin1*-transgenic or *Pin1*-deficient mice in the presence or absence of other oncogenes will be important to address the role of Pin1 in oncogenesis in vivo.

Pin1 thus functions at multiple steps in oncogenic signaling pathways as an 'oncogenic catalyst' (Fig. 2). It collaborates with Ras/JNK signaling to increase the transcriptional activity of Jun towards cyclin D1 (Wulf et al., 2001). It also activates β-catenin, which can induce the transcription of the cyclin D1 gene, *Jun* and *Myc* (Behrens et al., 1996; He et al., 1998; Mann et al., 1999; Molenaar et al., 1996; Ryo et al., 2002; Tetsu and McCormick, 1999). In addition, Myc can enhance cyclin D1 function by inducing Cdk4 expression (Hermeking et al., 2000) and also directly induce *E2F* family genes (Leone et al., 2000; Sears et al., 1997). These molecules act synergistically to regulate cyclin D1 and E2F function. Finally, *Pin1* itself is further upregulated by E2F activation in a positive feedback loop (Ryo et al., 2002) (Fig. 2). The amplification of this positive feedback pathway may play a role in aberrant cell proliferation and oncogenesis.

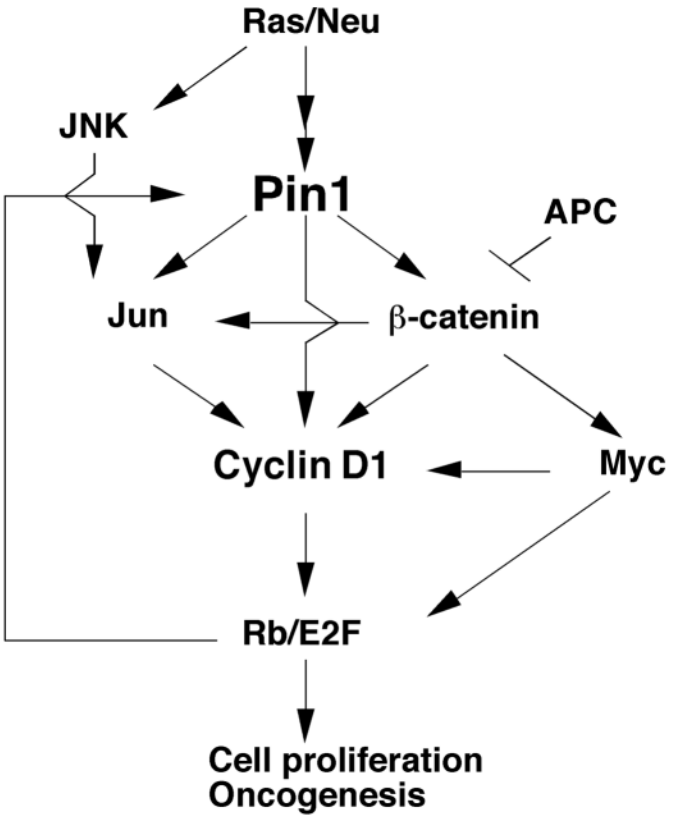

Fig. 2. Pin1 functions as a critical catalyst for integrating multiple oncogenic signaling pathways. Pin1 gene expression is induced by growth factor signaling through Neu and Ras signaling. Ras signaling induces JNK/SAPK activity to phosphorylate Jun. Subsequently, Pin1 binds to and isomerizes phosphorylated Jun to enhance its transcriptional activity. In parallel, Pin1 activates the β-catenin pathway by preventing β-catenin binding to APC, which can induce Jun gene expression. These signaling cascades eventually lead to an increase in cyclin D1 transcription. Furthermore, Pin1 also directly binds to and stabilizes cyclin D1 protein. In addition, Pin1 can induce the c-Myc gene through the activation of the β-catenin pathway, which can then enhance cyclin D1 function by inducing Cdk4 gene expression and/or directly activate E2F family genes. Finally, E2F can induce Pin1 expression in a positive feedback loop involving the cyclin D1/E2F pathway.

Therapeutic implications

Several lines of evidence suggest that inhibition of Pin1 can suppress oncogenesis, offering an attractive option for anti-cancer therapy. First, Pin1 has an extraordinarily high substrate specificity and well-defined active site (Lu et al., 1999b; Ranganathan et al., 1997; Shen et al., 1998; Verdecia et al., 2000; Yaffe et al., 1997). Historically, it has been much easier to develop inhibitors specific for an enzyme, such as Pin1, than for a non-enzymatic protein, such as cyclin D1. Second, Pin1 is overexpressed in cancer cells and can potentiate the function of some oncogenes (Ryo et al., 2002; Ryo et al., 2001; Wulf et al., 2001). Third, overexpression of Pin1 can confer transforming properties on mammary epithelial cells and also enhance transformed phenotypes of mammary epithelial cells induced by Neu and Ras (Ryo et al., 2002). Fourth, depletion of Pin1 using antisense PIN1 or dominant negative Pin1 causes cancer cells to enter mitotic block and apoptosis in transient transfection (Lu et al., 1996; Lu, P. J. et al., 2002; Rippmann et al., 2000). Recent reports that Pin1 associates with the anti-apoptotic protein Bcl-2 in mitosis (Basu et al., 2002; Pathan et al., 2001) suggest that apoptosis induced by Pin1 inhibition may occur via modulation of Bcl-2 function, although the biological significance of this interaction remains to be elucidated. Fifth, inhibition of Pin1 by stable expression of dominant negative Pin1 suppresses the transformed phenotypes induced by Ras/Neu, which can be reversed by the constitutively active cyclin D1 mutant that is resistant to Pin1 inhibition (Ryo et al., 2002). Finally, since *Pin1*-knockout mice do reach adulthood despite some cell proliferative abnormalities, especially in old age (Fujimori et al., 1999; Liou et al., 2002), an anti-Pin1 therapy might not have general toxic effects.

The feasibility of therapeutic inhibition of Pin1 has not yet been explored. In contrast to cyclophilins and FK506-binding proteins, where highly specific inhibitors are well characterized and widely used clinically (Fischer, 1994; Hunter, 1998; Schreiber, 1991), the only known Pin1 inhibitor is Juglone (Hennig et al., 1998). Juglone covalently inactivates a unique cysteine residue in the active site of Pin1-type and parvulin-type isomerases. Juglone has some anti-cancer activity and has been used as a Pin1 inhibitor in several studies in cells (Chao

et al., 2001; He et al., 2001; Rippmann et al., 2000). However, given that Juglone potently inhibits many other proteins and enzymes (Chao et al., 2001; Duhaiman, 1996; Munday and Munday, 2000; Muto et al., 1987), it is unlikely to be Pin1 specific in the cell. Therefore, there is a need for the development of Pin1-specific inhibitors. In addition to providing powerful tools for dissecting Pin1 function in vivo, such Pin1-specific inhibitors may open a new avenue for anticancer treatment. They may themselves be highly effective anticancer drugs or become valuable adjuncts to established chemotherapeutic regimen.

Conclusions and perspectives

It has become evident that phosphorylation-dependent prolyl isomerization is a previously uncharacterized post-phosphorylation signaling mechanism in cell proliferation and transformation. Following phosphorylation induced by oncogenic signaling pathways, *Pin1* catalyzed prolyl-isomerization is able to induce conformational changes and thereby to regulate the function of phosphorylated proteins. Interestingly, the level and activity of Pin1 itself are also upregulated by oncogenic pathways. Therefore, Pin1 may function as a critical catalyst that amplifies and translates multiple oncogenic signaling mechanisms during oncogenesis. Inhibition of Pin1 may thus provide a unique way of disrupting oncogenic pathways and therefore become an appealing target for novel anticancer therapies. However, further experiments, including in vivo studies using Pin1-knockout and Pin1-transgenic mouse models in the presence or absence of other oncogenes are necessary to elucidate the function and regulation of Pin1 in cell growth regulation and oncogenesis.

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