Prolactin Regulates Cyclin D1 Promoter Activity via Serine-threonine Kinase PAK1 and Adapter Protein Nck

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1 Introduction

1.1 Prolactin in Human Breast Cancer

Prolactin (PRL), a hormone of the growth hormone/cytokine family, exerts both endocrine and autocrine/paracrine effects and functions in both reproduction and as a cytokine (Bernichtein et al., 2010; Ben-Jonathan et al., 2008). Accumulating evidence from a variety of sources demonstrate a link between PRL and breast cancer (Tworoger and Hankinson, 2006; Clevenger, 2003). It has been shown that high circulating levels of PRL increase the risk of breast cancer in women (Tworoger and Hankinson, 2006; Hankinson et al., 1999), PRL receptor (PRLR) is overexpressed in 95% of breast cancer cases and PRL receptor can be stabilized by oncogenic pathways (Swaminathan et al., 2008; Meng et al., 2004; Touraine et al., 1998; Clevenger et al., 1995; Ginsburg and Vonderhaar, 1995). The examination of transgenic mouse models provided experimental evidence that a sustained increase in the levels of circulating lactogenic hormones, in particular PRL, causes mammary cancer (Wenbo et al., 1997; Tornell et al., 1991). Moreover, human breast cancer cells are able to upregulate the local synthesis of PRL, suggesting that this hormone can act in an autocrine manner to promote the proliferation of neoplastic cells (Clevenger et al., 1995; Ginsburg and Vonderhaar, 1995).

Initiation of PRL signaling involves PRL binding to PRLR and activation of the tyrosine kinase JAK2 which, in turn, phosphorylates the PRLR. Phosphorylated tyrosines (Tyr) within the receptor and JAK2 recruit an array of effector and/or signaling proteins. The best identified target of JAK2 is a family of transcription factors termed Signal Transducers and Activators of Transcription (STATs). STATs exist within the cytoplasm in a latent or inactive state; they are recruited by cytokine receptor complexes through an interaction involving a phosphotyrosine (on the cytokine receptor and/or the associated JAK) and the SH2 domain of the STAT protein (Reich, 2007; Schindler et al., 2007; Lim and Cao, 2006). Three members of the STAT family participate in PRL signaling: STAT1, STAT3 and STAT5 (both A and B isoforms) (Schaber et al., 1998; DaSilva et al., 1996; Ball et al., 1988). Additionally, phosphorylation of STAT6 during pregnancy has also been recently demonstrated (Khaled et al., 2007). STAT5 was originally identified as mammary gland factor (Wakao et al., 1994) and is the major STAT activated by PRL. JAK2/STAT5 pathway mediates most PRL action in lobuloalveolar development and lactation. However, the role of this pathway in the development and progression of breast cancer is more complex. Current data support the concept of dual roles of STAT5 as promoter of mammary tumorigenesis, and as suppressors of the progression of established breast cancer (Tan and Nevalainen, 2008; Wagner and Rui, 2008). JAK2 phosphorylation of STATs leads to their dimerization and translocation into the nucleus where they bind to specific response elements (GAS sequence) in the promoter of target genes, including promoter for cyclin D1 gene. The human cyclin D1 promoter contains two consensus GAS sites at -457 and -224. PRL induces STAT5 binding to the more distal GAS site (GAS1) to enhance cyclin D1 promoter activity (Brockman et al., 2002). PRL also induces cyclin D1 promoter activity by removing a ubiquitous transcriptional factor Oct-1 from the GAS2 site in the cyclin D1 promoter (Brockman and Schuler, 2005). In addition, using mammary cells from JAK2 knockout mice, JAK2 has been shown to control expression of the cyclin D1 mRNA and regulate the accumulation of cyclin D1 protein in the nucleus by inhibiting signal transducers that mediate the phosphorylation and nuclear export of cyclin D1 (Sakamoto et al., 2007).
1.2 Cyclin D1 As a Breast Cancer Oncogene

Cyclins regulate progression through the cell cycle and dysregulated expression of cyclins and/or cyclin-dependent kinases can lead to aberrant cellular growth, proliferation and tumorigenesis. D-type cyclins (i.e., cyclin D1, D2, and D3) are regulators of the cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) and mediate the growth factor-induced progression through the G1 phase of the cell cycle (Sherr, 1995; Diehl, 2002). Activation of these kinases by D cyclins results in phosphorylation of retinoblastoma protein, leading to increased transcription of E2F-responsive genes, and subsequent mitosis. In addition, cyclin D1 regulates multiple other processes relevant to oncogenesis, including other actions in cell cycle progression, adhesion and migration, responses to DNA damage, protein synthesis, metabolism, and differentiation, in many cases, independently of CDK4/6 or its kinase activity (Arnold and Papanikolaou, 2005; Fu et al., 2004; Coqueret, 2002) (Musgrove et al., 2011). Cyclin D1 is the most extensively studied member of the D-type cyclins due to its suggested pivotal role as a protooncogene in a number of human malignancies including breast cancer ((Knudsen et al., 2006; Lee and Sicinski, 2006; Sutherland and Musgrove, 2004; Suzuki et al., 1999; Dickson et al., 1995).

Among regulators of the cell cycle, cyclin D1 is a strong candidate target of PRL signaling since females deficient in cyclin D1 exhibit impaired mammary gland development similar to STAT5 knockout mice (Fantl et al., 1995; Sicinski et al., 1995). PRL is thought to influence cell proliferation and growth by altering the expression of cyclins D1 and B1 (Brockman and Schuler, 2005; Brockman et al., 2002; Schroeder et al., 2002). In addition to cyclins D1 and B1, a significant increase in cyclins A and E expression has been also detected in many breast cancers (Megha et al., 1999; Keyomarsi and Pardee, 1993). The cyclin D1 gene is amplified or overexpressed in up to 50% of human breast cancers (Dickson et al., 1995; McIntosh et al., 1995), the overexpression of cyclin D1 in the mammary epithelium leads to the formation of tumors in transgenic mice after a latency of more than 1 year (Wang et al., 1994), and interference of its nuclear export and proteolytic degradation has been shown to accelerate mammary carcinogenesis (Lin et al., 2008). Moreover, the targeted ablation of cyclin D1 or the inhibition of its correct functional association with Cdk4/6 was suggested to completely prevent the onset of ErbB2-associated mammary cancer (Jeselsohn et al., 2010; Landis et al., 2006; Yu et al., 2001). Interestingly, two independent mouse models have been recently established to demonstrate that lack of cycline D1 was associated with a compensatory upregulation of cyclin D3 indicating that cyclin D1 is an important but not essential mediator of PRL-induced mammary proliferation although is critical for differentiation and lactation (Asher et al., 2012; Zhang et al., 2011). How we mentioned earlier, not all actions of cyclin D–CDK4/CDK6 depend on substrate phosphorylation. Indeed, in addition to promotion of cell proliferation, cyclin D1 has been shown to regulate multiple other processes relevant to oncogenesis independently of CDK4/6 or its kinase activity (Musgrove et al., 2011). One major non-catalytic function of the D-cyclins is transcriptional regulation. Cyclin D1 is tethered to the promoters of many genes during normal development, probably through interactions with various transcription factors. Thus, Sicinski and colleagues examining cyclin D1–associated proteins in mouse embryos determined that about one third of the identified proteins were transcription factors (Bienvenu et al., 2010). It is clear that understanding the mediators of PRL/cyclin D1 action in carcinogenesis will reveal potential sites for preventative and therapeutic interventions.
1.3 Serine-Threonine kinase PAK1 is involved in Breast Cancer Progression

We have recently linked PRL signaling to the serine-threonine kinase PAK1 and shown that JAK2 directly phosphorylates PAK1 (Rider et al., 2007) (Figure 1).

![Figure 1: Schematic diagram depicting PAK1. The N-terminus of PAK1 contains five PXXP motifs (yellow bars) of which the first two bind SH2 domain of Nck and Grb2, respectively. PBD (p21-binding domain) domain is responsible for PAK1 activation by Rac1-3, Cdc42, Chp, TC-10 and Wrch-1 (dark-green box, amino acids 67-113). The autoinhibitory domain (AID, blue box, amino acids 87-149) overlaps with PBD, it associates in trans with the kinase domain of PAK1 (orange box, amino acids 255-529). Non-classical proline-rich domain (light-green box, amino acids 182-203) associates with PAX/GIT proteins. Fourteen tyrosines of PAK1 and their sequences are shown below. Tyrosines 153, 201 and 285 (shown in red) are sites of JAK2 phosphorylation.](image)

PAK1 is a member of a conserved family of p21-activated serine-threonine kinases, and is important for a variety of cellular functions, including cell morphogenesis, motility, survival, mitosis and malignant transformation (for review Kumar et al., 2006; Zhao and Manser, 2005; Bokoch, 2003). The emerging roles of PAK1 in the regulation of multiple fundamental cellular processes have directed significant attention towards understanding how PAK1 activity is controlled. Autoinhibition of the PAK1 C-terminal catalytic domain by the N-terminal domain is a key mechanism of PAK1 regulation. Several layers of inhibition, involving dimerization and occupation of the catalytic cleft by contact between the N- and C-terminal domains, keep PAK1 kinase activity in check (Lei et al., 2000). Autoinhibition of PAK1 occurs in trans, meaning that the inhibitory domain of one PAK1 molecule interacts with the kinase domain of another PAK1 molecule (Parrini et al., 2002). Association of GTP-bound forms of Cdc42 and Rac1 with the PAK1 PBD/CRIB domain induces conformational changes in the N-terminal domain that no longer support its autoinhibitory function. In addition to Cdc42 and Rac1, PAK1 is activated by the binding of small GTPases, Rac2 and Rac3, as well as TC10, CHP and Wrch-1 proteins (Tao et al., 2001; Mira et al., 2000; Aronheim et al., 1998; Knaus and Bokoch, 1998; Neudauer et al., 1998; Manser et al., 1994). PAK1 is a predominantly cytoplasmic protein, but is activated upon recruitment to the cell membrane. PAK1 membrane localization occurs through interaction with adaptor proteins Nck, Grb2 and PIX, all of which are activated by ligation of growth-factor receptors (Zhao et al., 2000b; Daniels et al.,
PAK1 is overexpressed in breast cancer cell lines and tumor tissues express hyperactive PAK1 and its upstream regulator Rac3 (Mira et al., 2000). Activated PAK1 increased cell invasion of breast cancer cells and expression of a kinase-dead PAK1 mutant in the highly invasive breast cancer cell lines led to a reduction in invasiveness (Adam et al., 2000). Conversely, hyperactivation of the PAK1 pathway in the non-invasive breast cancer cell line MCF-7 promotes cell migration and anchorage-independent growth (Vadlamudi et al., 2000). Recently PAK1 has been shown to phosphorylate dynein light chain 1 (DLC1) that plays a critical role in tumorigenic phenotypes of DLC1 in breast cancer cells (Vadlamudi et al., 2004). Thus, PAK1 has become one of the focal points in the investigation into the mechanism and onset of human breast cancer.

PAK1 has also been implicated in regulation of cyclin D1 gene expression. Overexpression of catalytically active PAK1 T423E in MCF7 cells leads to cyclin D1 expression while overexpression of PAK1 lacking the nuclear localization signals does not (Holm et al., 2006; Rayala et al., 2006; Balasenthil et al., 2004). Reducing PAK1 expression by PAK1-siRNA is accompanied by a significant reduction of cyclins D1 and B1 expression (Balasenthil et al., 2004). PAK1 has a well-established role in the nucleus, where it associates with chromatin, phosphorylates histone H3 and several transcription factors and transcriptional coregulators (Park et al., 2007; Singh et al., 2005; Li et al., 2002, for review Rayala and Kumar, 2007).

Understanding the mechanism by which PRL stimulates mitogenesis and how it interacts with other factors important in breast cancer may lead to improved diagnostic assays and therapeutic approaches. In this study we have linked PRL and PAK1 as the JAK2 substrate to the stimulation of cyclin D1 promoter activity. We have proposed two mechanisms by which PRL regulates cyclin D1 promoter activity.
The first is a positive effect that depends on the PRL-dependent phosphorylation of three tyrosines on PAK1 and the presence of PAK1 nuclear localization signals. The second is a counter-regulatory mechanism that involves the interaction between PAK1 and adapter protein Nck, which keeps the Nck-PAK1 complex in the cytoplasm.

2 Results

2.1 Prolactin-activated Tyrosyl Phosphorylated PAK1 Stimulates Cyclin D1 Promoter Activity

Both PAK1 and prolactin have previously been implicated in the regulation of cyclin D1 promoter activity (Balasenthil et al., 2004; Brockman et al., 2002). Since we have recently shown that PRL causes tyrosyl phosphorylation of PAK1 by JAK2 kinase (Rider et al., 2007), we decided to investigate whether tyrosyl phosphorylation of PAK1 is important for cyclin D1 regulation in response to PRL. First, we measured the induction of cyclin D1 promoter activity in T47D cells treated with or without PRL. As shown in Figure 2A, T47D cells transfected with a human cyclin D1 promoter-luciferase construct increased luciferase expression in response to PRL as expected. Second, co-transfection of T47D cells with luciferase construct and PAK1 WT results in a 4.6-fold increase in luciferase expression in the absence of PRL that corresponds to previously published data (white bars in Figure 2B) (Balasenthil et al., 2004). Interestingly, treatment of the PAK1 WT-expressing cells with PRL causes a 14-fold increase in luciferase expression as compared with the cells not expressing PAK1 WT and treated with PRL (black bars in Figure 2B). Overexpression of PAK1 lacking the three phosphorylated tyrosines (PAK1 Y3F) which are sites of JAK2 phosphorylation, reduced PAK1’s effect on cyclin D1 promoter activity by 55% compared to PAK1 WT in the presence of PRL. These data suggest that Tyr(s) 153, 201 and 285 of PAK1 are required for maximal cyclin D1 promoter activity in response to PRL.

2.2 PAK1 Shuttles between the Cytoplasm and Nucleus and PRL Promotes PAK1 Nuclear Accumulation

Data from the literature suggest that PAK1 translocates into the nucleus in response to EGF (Singh et al., 2005) and we wished to investigate the potential significance of PAK1 nuclear localization for cyclin D1 regulation. We first studied whether PRL can stimulate nuclear translocation of PAK1. Figure 3 indicates that treatment of T47D cells with PRL for 24h caused nuclear accumulation of endogenous PAK1. Interestingly, extended incubation of T47D cells with PRL up to 48h led to re-distribution of PAK1 back to the cytoplasm. These immunofluorescence data were confirmed by fractionation assay demonstrating the presence of PAK1 in both cytoplasmic and nuclear fractions before PRL treatment, elevated levels of PAK1 in the nuclear fraction 24h after PRL addition and a decrease in nuclear PAK1 after 48h (not shown).

In order to investigate the role of the three sites of JAK2-dependent tyrosyl phosphorylation of the PAK1 molecule in nuclear translocation, we overexpressed either PAK1 WT or PAK1 Y3F in T47D cells, treated them with or without PRL to activate JAK2 and defined the amount of cells with nuclear PAK1 (Figure 4A and B). There were significantly more cells with intranuclear PAK1 WT after PRL treatment than without PRL, while there was no PRL-dependent difference in localization of PAK1 Y3F mutant, suggesting that these three tyrosines may play a role in PAK1 nuclear translocation. We have also seen significant PAK1 translocation into the nucleus when we overexpressed PAK1 WT with JAK2 in COS-7 and MCF-7 cells (Figure 5, left two bars in each plot).
Figure 2: Prolactin stimulates cyclin D1 promoter activity through tyrosines 153, 201 and 285 of PAK1. T47D cells were transfected with cyclin D1-luciferase reporter (A) or cotransfected with cyclin D1-luciferase reporter with vector, PAK1 WT or PAK1 Y3F (B). The cells were treated with (black bars) or without (white bars) 500 ng/ml of prolactin for an additional 24h, lysed, and luciferase activity was measured. Luciferase activity was normalized with β-galactosidase activity. Bars represent mean ±S.E., *, p <0.05, n=3.

Figure 3: Prolactin causes translocation of endogenous PAK1 into nucleus. T47D cells were deprived of serum for 24h and treated with or without 500 ng/ml PRL for 0, 24 or 48h. Endogenous PAK1 was subjected to confocal immunofluorescence with αPAK1 antibody. Scale bar, 50 μm.
Figure 4: Tyrosyl phosphorylation of PAK1 is required for translocation of PAK1 into nucleus in response to PRL. (A) T47D cells were transfected with either PAK1 WT or PAK1 Y3F. The cells were serum deprived for 24h, treated with or without 500 ng/ml of PRL for an additional 24h and PAK1 was immunolocalized with αPAK1 antibody. Scale bar, 25 µm. (B) The percentage of cells with PAK1 nuclear localization was counted and plotted. 100 PAK1-expressing cells were assessed for PAK1 or PAK1 Y3F immunolocalization in each experiment for each type of treatment. Bars represent mean ±S.E., *, p<0.05, n=3.

Since the maximal amount of nuclear endogenous PAK1 was observed 24h but not 48h after PRL treatment, we hypothesize that PAK1 may shuttle between the nucleus and the cytoplasm. To test this hypothesis, we treated T47D cells with Leptomycin B (LMB), a specific inhibitor of Crm1-dependent nuclear export. Indeed, LMB treatment lead to nuclear accumulation of overexpressed PAK1 WT in T47D, MCF-7 and COS-7 cells indicating that nucleo-cytoplasmic shuttling of PAK1 was happening and that this occurred in a cell-type independent manner (Figure 5).

2.3 Effect of Nuclear Localization Signals and Tyrosyl Phosphorylation of PAK1 on Cyclin D1 Promoter Activity

To further implicate a regulatory role of PAK1 tyrosyl phosphorylation in nuclear localization and the regulation of cyclin D1 transcription, we used a previously described PAK1 mutant in which three nuclear localization signals (NLS) have been mutated by replacing the three basic lysine residues with alanines (amino acids 48-51 for NLS1, 243-245 for nLS2 and 267-269 for NLS3) (Singh et al., 2005). Because overexpression of this PAK1 mutant lacking the three functional nuclear localization signals (PAK1 mutNLS) decreased but did not eliminate augmentation of cyclin D1 promoter activity compared to PAK1 WT (Holm et al., 2006), we hypothesize that mutation of Tyr(s) 153, 201 and 285 in PAK1 mut-NLS will further reduce PRL-induced activation of cyclin D1 promoter. To test this, we transiently expressed PAK1 mutNLS or PAK1 mutNLS Y3F mutants in T47D cells, treated the cells with or without PRL and performed experiments as described above. As shown in Fig.6A, expression of PAK1 mutNLS decreased both PRL-dependent and PRL-independent cyclin D1 transcription activity by 46% i. e. to a similar level caused by expression of PAK1 Y3F mutant (47% inhibition in this experiment). Expression of PAK1 mutNLS Y3F significantly decreased the effect of PRL on cyclin D1 promoter activity by 68%,
Figure 5: PAK1 shuttles between cytoplasm and nucleus. PAK1 alone (T47D cells) or PAK1 and JAK2 (COS-7 and MCF-7 cells) were overexpressed in the indicated cells. The cells were incubated with Leptomycin B (LMB) for 8h and processed for immunolocalization of PAK1 with αPAK1 antibody. T47D cells were treated with 500 ng/ml of PRL for 48h before LMB was added. The percentage of cells with PAK1 nuclear localization was counted and plotted. 100 PAK1-positive (for T47D cells) and both PAK1- and JAK2-positive (for COS-7 and MCF-7 cells) cells were assessed for PAK1 immunolocalization in each experiment for each type of treatment. Scale bar, 25 µm. Bars represent mean ±S.E., *, p<0.05, n=3.
Figure 6: Effect of PAK1 nuclear localization signals and tyrosyl phosphorylation on cyclin D1 promoter activity. T47D cells were cotransfected with cyclin D1-luciferase reporter with either vector, PAK1 WT, PAK1 mutNLS (three nuclear localization signals mutated), PAK1 Y3F or PAK1 mutNLS Y3F. The cells were serum deprived for 24h, treated with (black bars) or without (white bars) 500 ng/ml of prolactin for an additional 24h, lysed, and luciferase activity was measured. Luciferase activity was normalized with β-galactosidase activity. Bars represent mean ±S.E., *, p <0.05, n=3. The expression levels of PAK1 WT and PAK1 mutants are indicated (A). Total cell lysates, cytosolic (C) and nuclear (N) fractions of T47D cells transfected with PAK1 WT or PAK1 mutants and treated with or without PRL were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with αPAK1, αpaxillin as a cytosolic marker and αRARα as a nuclear marker (B).
suggesting that both nuclear localization and tyrosyl phosphorylation of PAK1 are required for the maximal effect of PRL on cyclin D1 promoter activity. Figure 6B indicates that treatment of T47D cells with PRL caused nuclear accumulation of overexpressed PAK1 WT but not PAK1 mutNLS, PAK1 Y3F or PAK1 mutNLS Y3F mutants.

2.4 Nck Regulates PAK1 Nuclear Localization and Inhibits PAK1-stimulated Cyclin D1 Promoter Activity

In our search for additional PAK1-dependent mechanisms of cyclin D1 promoter activation, we investigated a role for the adapter protein Nck since Nck is a known binding partner of PAK1 (Bokoch et al., 1996; Galisteo et al., 1996), that is known to shuttle between the cytoplasm and nucleus (Kremer et al., 2007). We first investigated the effect of Nck expression on PAK1 nuclear relocation in response to PRL by overexpressing PAK1 WT alone, Nck alone or PAK1 with Nck together and treating T47D cells with or without PRL. The number of the cells with nuclear PAK1 and the number of cells with nuclear Nck were counted and plotted (Figure 7 B, C). As illustrated in Figure 7A-C, Nck retained PAK1 in the cytoplasm (2 left white bars in Figure 7B) and inhibited PAK1 nuclear translocation in response to PRL (2 left black bars in Figure 7B). This effect was partially inhibited by expressing PAK1 Y3F instead of PAK1 WT, but only for PRL-untreated cells (PAK1 WT+Nck vs. PAK1 Y3F+Nck without PRL, white bars in Figure 7B). We did not see a significant difference between the cells expressing the same constructs but treated with PRL (PAK1 WT+Nck vs. PAK1 Y3F-Nck with PRL, black bars in Figure 7B).

These data suggest that the three tyrosines on PAK1 may play a role in localization of the PAK1-Nck complex, but this effect is not affected by PRL-dependent tyrosyl phosphorylation. Interestingly, the percentage of cells in which Nck localized to both the cytoplasmic and nuclear compartments was decreased by up to 45% when it was co-expressed with PAK1 (Figure 7A and C). This effect was independent of PRL treatment (Figure 7A and C). These data suggest that Nck sequesters PAK1 in the cytoplasm and that it stays in the cytoplasm itself when complexed with PAK1. Tyrosyl phosphorylation of PAK1 on the three tyrosines assessed does not play a role in this process (Figure 7A and C, last two bars).

Data from the experiments with the luciferase-cyclin D1 promoter construct demonstrated that co-expression of Nck with PAK1 WT strongly inhibited (by 95%) the impact of PAK1 on cyclin D1 promoter activity both in the presence and absence of PRL (Figure 8A). This inhibition was much stronger than that caused by expression of PAK1 Y3F (by 60%).

To study further whether the inhibitory effect of Nck on PAK1-induced cyclin D1 promoter activity was relieved by disruption of Nck-PAK1 binding, we used two mutants: Nck W143R mutant has a mutation in the second SH3 domain and fails to bind to PAK (Zhu et al., 2010), and PAK1 P13A mutant, a mutant that is unable to interact with Nck (Bokoch et al., 1996; Galisteo et al., 1996). Our co-immunoprecipitation experiments confirmed that only PAK1 WT and Nck WT bound to each other in vivo while PAK1 P13A and Nck W143R did not (Figure 9B). Data in Fig. 9A demonstrate that PAK1 P13A and PAK1 WT augment cyclin D1 promoter activity. Mutation of tryptophan 143 to arginine in Nck had no effect on cyclin D1 promoter activity as compared with Nck WT. Expression of both PAK WT and Nck WT strongly inhibited cyclin D1 promoter activity as described before. However, disruption of Nck-PAK1 binding by expression of mutated PAK1 and Nck partially but significantly relieved the repression of PRL-dependent stimulation, induced by Nck WT (Figure 9A). These data confirm that a functional Nck-PAK1 complex is required for optimal regulation of cyclin D1 promoter activity.
Figure 7: Nck retains PAK1 in the cytoplasm. T47D cells were either transfected with PAK1 WT or Nck, or co-transfected with Nck and either PAK1 WT or PAK1 Y3F, serum deprived for 24h, treated with or without 500 ng/ml of PRL for an additional 24h. PAK1 and Nck were immunolocalized with αPAK1 or αNck correspondingly. Scale bar, 25 µm. (A). The percentage of cells with PAK1 (B) or Nck (C) nuclear localization was counted and plotted. 100 PAK1-expressing or both PAK1- and Nck-expressing cells were assessed for PAK1 or Nck immunolocalization in each experiment for each type of treatment. Bars represent mean ±S.E., *, p<0.05, n=3.
Nck blocks the amplifying effect of PAK1 on cyclin D1 promoter activity. (A) T47D cells were cotransfected with cyclin D1-luciferase reporter, and either PAK1 WT, PAK1 and Nck, PAK1 Y3F or PAK1 Y3F and Nck. The cells were serum deprived for 24h, treated with (black bars) or without (white bars) 500 ng/ml of PRL for an additional 24h, lysed, and luciferase activity was measured. Luciferase activity was normalized with β-galactosidase activity. Bars represent mean ±S.E., *, p <0.05 compared with cells expressing PAK1 WT and untreated with PRL, n=3. (B) Whole-cell lysates of T47D cells transfected with PAK1 WT, PAK1 Y3F and Nck were subjected to αPAK1 and αNck Western blotting. The expression levels of PAK1 WT, PAK1 Y3F and Nck are indicated.
3 Discussion

The effect of PRL on regulation of the cell cycle progression increases our understanding of the mechanism by which PRL may stimulate growth during mammary development. Furthermore, in an abnormal genetic or environmental context, this action may contribute to mammary carcinogenesis and may point toward potential targets for pharmacological intervention in this process. Here we introduce the serine-threonine kinase PAK1 as a possible target in the PRL-dependent signaling pathway leading to cyclin D1 activation.

PAK1 has been suggested to serve as the key effector for Rac1 activation of cyclin D1 (Westwick et al., 1997). Later, PAK1 was shown to activate cyclin D1 in vivo and in vitro (Balasenthil et al., 2004). Overexpression of both wild type and catalytically active PAK1 T423E in different cell lines led to increased cyclin D1 promoter activity, and the overexpression of PAK1 T423E in MCF-7 cells also elevated levels of cyclin D1 mRNA, protein and nuclear accumulation of cyclin D1. Reducing PAK1 expression by PAK1-siRNA or overexpression of dominant negative PAK1 were accompanied by a significant reduction of cyclin D1 expression (Balasenthil et al., 2004). The same authors demonstrated that hyperplastic mammary glands from PAK1 T423E transgenic mice exhibited increased expression of cyclin D1 as compared to the wild type mice. The authors proposed a model wherein PAK1 regulation of cyclin D1 expression involves an NFkB-dependent pathway (Balasenthil et al., 2004). Merlin, the NF2 tumor suppressor gene product, has been proposed as a negative regulator of PAK1-stimulated cyclin D1 promoter activity by inhibition of PAK1 activity (Xiao et al., 2005). Nheu et al. (2004) demonstrated that PAK activity is essential for rennin-angiotensin system-induced upregulation of cyclin D1 (Nheu et al., 2004). All of the aforementioned studies explained the effect of PAK1 on cyclin D1 activity by the serine-threonine kinase activity of PAK1.

However, many of the effects of PAK1 seem to be independent of its kinase activity but dependent on protein-protein interaction. Thus, the kinase inhibitory domain of PAK1 (KID) induced a cell cycle arrest and inhibition of cyclin D1 and D2 expression. More importantly, this arrest could not be rescued by the expression of activated PAK1 T423E demonstrating that KID-induced cell cycle arrest occurs independently of PAK1 kinase activity (Thullberg et al., 2007).

Here we linked upstream PRL-triggered signaling via JAK2 tyrosine kinase to downstream PAK1 which is a JAK2 target. We have previously demonstrated that PAK1 is a novel binding partner and a substrate of JAK2 in response to PRL activation, and identified three tyrosines (Tyr(s) 153, 201 and 285) of PAK1 that are phosphorylated by JAK2 (Rider et al., 2007). Here we have shown that PAK1, in response to PRL, causes an increase of cyclin D1 promoter activity and mutation of three tyrosines (Tyr 153, 201, 285) inhibit this amplifying effect by 55%. In an attempt to find a mechanism of this pTyr-PAK1 action, we noticed that PRL causes translocation of PAK1 into the nuclei. Nuclear translocation of PAK1 in response to EGF has been previously described (Singh et al., 2005; for review Rayala and Kumar, 2007). Thus, endogenous PAK1 localizes in the nucleus in 18-24% of the interphase MCF-7 cells and directly phosphorylates histone H3 (Li et al., 2002). PAK1 associates with the promoter of PFK-M gene and stimulates PFK-M expression, and also with a portion of the NFAT1 gene and represses expression of this gene (Singh et al., 2005). In addition, increased levels of nuclear PAK1 were linked to intrinsic tamoxifen resistance of breast cancer cells (Holm et al., 2006; Li et al., 2002). We extended these findings and demonstrated that PAK1 shuttles between the cytoplasm and the nucleus in different cell lines including T47D, COS-7 and MCF-7. Furthermore, we have shown that PRL-dependent PAK1 nuclear translocation depends on Tyr 153, 201 and 285 since the PAK1 Y3F mutant does not translocate...
into the nucleus in response to PRL. Three nuclear localization signals have been mapped on PAK1, and PAK1 lacking these three functional NLS (PAK1 mutNLS) fails to translocate into the nucleus in response to EGF (Singh et al., 2005). We have shown here that PAK1 mutNLS exhibited 46% less impact on cyclin D promoter activity as compared to PAK1 WT. We hypothesized that by eliminating both PAK1 tyrosine phosphorylation and functional NLSs, we would completely inhibit PRL-dependent amplification of cyclin D1 promoter activity. However, PAK1 mutNLS Y3F exhibited only 68% inhibition, suggesting that both the three nuclear localization signals and three tyrosines which are phosphorylated by JAK2 in response to PRL contribute to but are not exclusively required for the PRL-dependent induction of cyclin D1 promoter activity. Why do PAK1 mutNLS and PAK1 mutNLS Y3F, which both retain in the cytoplasm, still have an amplifying effect on cyclin D1 activity? PAK1 may activate cyclin D1 promoter activity in response to PRL via multiple mechanisms. For example, PAK1 phosphorylates specific cytoplasmic proteins that can directly or indirectly regulate cyclin D1 promoter activity. In this context it is interesting to note that, although PRL signals via STAT5 to the distal GAS1 binding sites in the cyclin D1 promoter, co-expression of dominant negative STAT5A and PAK1 WT showed no effect on cyclin D1 promoter activity (Brockman and Schuler, 2005; Balasenthil et al., 2004; Brockman et al., 2002). We are currently investigating which regulatory elements of cyclin D1 promoter are affected by PRL-dependent PAK1 tyrosyl phosphorylation.

In attempt to find another mechanism that can regulate the action of PRL on the cyclin D1, we focused on Nck for several reasons. First, adapter protein Nck is a binding partner of PAK1 (Bokoch et al., 1996; Galisteo et al., 1996). Nck W143R mutant with a mutation in the second SH3 domain, fails to bind to PAK (Zhu et al., 2010) and the PAK1 P13A mutant is unable to interact with Nck (Bokoch et al., 1996; Galisteo et al., 1996). Second, Nck is present in both the cytoplasm and the nucleus (Lawe et al., 1997). Nck rapidly accumulates in the nucleus after the introduction of DNA damage (Kremer et al., 2007). In agreement with Lawe et.al., who showed that nuclear localization of Nck does not depend on growth factor stimulation (Lawe et al., 1997), we have shown here that PRL also does not cause Nck nuclear translocation and that around 65% of cells contain nuclear Nck regardless of PRL treatment. However, when we co-expressed both Nck and PAK1, both molecules were mostly retained in the cytoplasm. This effect was especially dramatic for PAK1, since 3-fold fewer cells had nuclear PAK1 as compared with cells without Nck co-expression. More importantly, Nck abolished the ability of PRL to induce PAK1 nuclear translocation. These data suggest that Nck can sequester PAK1 in the cytoplasm. This sequestering has a physiological role since co-expression of both PAK1 and Nck inhibits the amplifying effect of PRL-induced PAK1 on cyclin D1 promoter activity (95% inhibition). This inhibition was partially abolished by disruption of the PAK1-Nck binding by using either PAK1 P13A mutant, Nck W143R mutant, or both. This partial inhibition implies the presence of Nck-PAK1-interaction-independent mechanisms that affect cyclin D1 promoter activity. Nck is a common target for a variety of growth factor receptors and becomes phosphorylated on serine, threonine and tyrosine residues after growth factor stimulation (Chou et al., 1992; Li et al., 1992; Park and Rhee, 1992). Nck is implicated in the regulation of different signal transduction pathways including c-Jun N-terminal kinase (JNK) and mixed lineage kinase 2 (MLK2) pathways (Miyamoto et al., 2004; Poitras et al., 2003; Murakami et al., 2002; Becker et al., 2000; Stein et al., 1998; Su et al., 1997). Furthermore, nuclear Nck is essential for activation of p53 in response to UV-induced DNA damage (Kremer et al., 2007). Transcriptional regulation of the cyclin D1 gene as a complex and many different transcription factors have been identified that regulate the cyclin D1 promoter (reviewed in Wang et al., 2004). The regulation of cyclin D1 by integrin signaling is well-
documented (reviewed in Musgrove, 2006) and Nck is an important member of focal adhesions and integrin-dependent pathway (reviewed in Buday et al., 2002).

It is possible that there are mechanisms other than retention of Nck-pTyr-PAK1 complex in the cytoplasm, to regulate the cyclin D1 promoter activity. Nck is a binding partner of PTP-PEST (a cytosolic protein tyrosine phosphatase) (Zhao et al., 2000a). PTP-PEST has been shown to dephosphorylate PRL-activated JAK2 in vitro (Horsch et al., 2001). We can speculate that Nck brings PTP-PEST to the PRL-induced JAK2-PAK1 complex that may lead to dephosphorylation and inactivation of JAK2. Inactive JAK2 cannot tyrosyl phosphorylate PAK1 which leads to inability of PAK1 to enhance cyclin D1 activation. Another possible common target which binds to both Nck and JAK2 is the ubiquitin ligase c-Cbl, which is a negative regulator of various signaling pathways. C-Cbl becomes tyrosyl-phosphorylated after stimulation of a wide variety of receptors including the PRL receptor (Hunter et al., 1997). The negative regulation of PRL signaling by c-Cbl is confirmed by observations that c-Cbl repression leads to enhanced JAK2/STAT activation while c-Cbl overexpression results in increased ubiquitination and proteosomal degradation of STAT5 (Goh et al., 2002; Wang et al., 2002). Since c-Cbl binds to Nck (Wunderlich et al., 1999; Rivero-Lezcano et al., 1994), we can speculate that Nck may bring c-Cbl to the PRL-induced JAK2-PAK1 complex, thus leading to proteosomal degradation of JAK2 followed by attenuation of pTyr-PAK1’s effect on cyclin D1 promoter activity. Nck also binds to SOCS-3 and recruits Nck to the plasma membrane (Sitko et al., 2004). SOCS-3 negatively regulates PRL signaling by interacting with phosphorylated PRLR leading to JAK2 suppression and, probably, by directly interacting with JAK2 which has been shown for the erythropoietin receptor (Dif et al., 2001; Sasaki et al., 2000; Tomic et al., 1999). It would be attractive to speculate that Nck-SOCS-3 complex is recruited to the plasma membrane to bind to the PRLR leading to inactivation of JAK2 and decreased pTyr-PAK1’s activity toward cyclin D1 promoter. We should, however, note that we have not seen relocation of Nck to the plasma membrane in response to PRL. It is possible that the redistribution of Nck to the plasma membrane occurs shortly after ligand treatment (for example, in 30 min as it was described for PDGF treatment (Sitko et al., 2004)) while long-term treatment of PRL (24 h in the current research) has no effect on the predominantly intranuclear localization of Nck (Figure 7).

To summarize, Figure 10 shows an overall view of mechanisms of cyclin D1 promoter activity regulation by tyrosyl phosphorylated PAK1 in response to PRL. First, PRL binds to the PRL receptor and activates JAK2 which tyrosyl phosphorylates PAK1 on three tyrosines. Tyrosyl phosphorylated PAK1 translocates into the nucleus where it stimulates cyclin D1 promoter activity. Both tyrosyl phosphorylation of PAK1 and the three intact NLSs are required for this maximal effect of PRL on cyclin D1 because PAK1 mutNLS Y3F mutant has 68% reduced ability to activate the cyclin D1 promoter. However, the more critical mechanism for PAK1-dependent regulation of cyclin D1 activation is the formation of the PAK1-Nck complex in the cytoplasm. This complex retains PAK1 in the cytoplasm which leads to the inhibition of the amplifying effect of PAK1 on cyclin D1 promoter activity in response to PRL. Which protein(s) can regulate the formation of the Nck-PAK1 complex and what kind of role the phosphorylated tyrosines on PAK1 play are currently under our investigation.

It is of note that both PRL and PAK1 are oncogenic. Considering that cyclin D1 promoter activity is positively regulated by tyrosyl phosphorylated PAK1 in a PRL-dependent manner, we may speculate that the PRL-activated JAK2/PAK1 axis plays a role in breast cancer promotion. Whether JAK2-dependent phosphorylation of PAK1 plays a role in normal cells and in other types of cancer requires future investigation.
Figure 9: PAK1-Nck binding is required for the effect of Nck on cyclin D1 promoter activity. (A) T47D cells were cotransfected with cyclin D1-luciferase reporter and cDNAs encoding the indicated proteins and treated as in Figure 7A. Luciferase activity was normalized with β-galactosidase activity. Bars represent mean ±S.E., *, p <0.05 compared with cells expressing PAK1 WT and untreated with PRL, n=3. (B) Nck WT is co-immunoprecipitated with PAK1 WT (lane 5) but not with PAK1 P13A (lane 6). Nck W148R is co-immunoprecipitated neither with PAK1 WT (lane 7) nor with PAK1 P13A (lane 8). HA-tagged Nck was immunoprecipitated with αHA from T47D cells overexpressing the indicated proteins and immunoblotted with the indicated antibodies. The light bands in lanes 1 and 3 in the αNck blot represent endogenous Nck.
Figure 10: PAK1 regulates prolactin-dependent cyclin D1 promoter activity by two distinct mechanisms. In response to cytokines such as prolactin, PAK1 is tyrosyl phosphorylated by JAK2 and translocates into the nucleus where it stimulates cyclin D1 promoter activity. The nuclear localization signals (NLS) and the three tyrosines of PAK1 are sufficient but not required for this PAK1 function since deletion of these three phosphorylated tyrosines (PAK1 Y3F) and mutation of the three NLSs (PAK1 mutNLS) decreased PAK1 WT activity on cyclin D1 promoter activity by 55% and 46%, respectively. The double mutant of PAK1 (mutNLS Y3F) decreased PAK1 WT activity on cyclin D1 promoter activity by 68%. Another mechanism regulating the PAK1 function is Nck-PAK1 binding, since co-expression of PAK1 and Nck inhibited PAK1-dependent stimulation of cyclin D1 promoter activity by 95%. We propose that the Nck-PAK1 complex sequesters both molecules in the cytoplasm, thereby abolishing the amplifying effect of PAK1 on the prolactin-induced activation of cyclin D1.

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