Cdc37 Enhances Proliferation and Is Necessary for Normal Human Prostate Epithelial Cell Survival

Steven R. Schwarze, Vivian X. Fu, and David F. Jarrard

Department of Surgery, Division of Urology, University of Wisconsin Medical School, Molecular and Environmental Toxicology and the University of Wisconsin Comprehensive Cancer Center, Madison, Wisconsin 53972

ABSTRACT

Cdc37 is a co-chaperone protein that targets several immature client kinases to Hsp90 for proper folding. Cdc37 up-regulation is a common early event in localized human prostate cancer. Although targeted overexpression in mice leads to prostate epithelial cell hyperplasia, the effect of Cdc37 dysregulation in human prostate cells is unclear. In this study, we examine the role of Cdc37 in the growth regulation of normal prostate epithelial cells using a unique human model system. We demonstrate that Cdc37 overexpression drives proliferation, whereas loss of Cdc37 function arrests growth and leads to apoptosis. With increased Cdc37 expression, molecular analysis of Cdc37 client pathways demonstrates enhanced Raf-1 activity, greater Cdk4 levels, and reduced expression of the cyclin-dependent kinase inhibitor p16/CDKN2. To further investigate these downstream pathways, enhanced Raf-1 or Cdk4 activities were selectively induced in human prostate epithelial cells. Raf-1 activation inhibited proliferation and generated an enlarged, flattened morphology. Induction of Cdk4 activity using cyclin D1 overexpression, however, was sufficient to promote proliferation. These data indicate that Cdc37 induces proliferation and is critical for survival in human prostate epithelial cells. These alterations in cell division and survival may be important in the development and progression of early prostate cancer.

INTRODUCTION

Cdc37 is a co-chaperone protein that targets and activates multiple protein kinases. These interactions are important for a number of mitogenic signaling pathways. As a critical component of cell cycle control, Cdc37 dysregulation has been implicated in the development of cancer. Recently human prostate tissue specimens were surveyed for Cdc37 expression and increased immunoreactivity was found in all specimens analyzed when compared with normal tissues (1). Cdc37 overexpression was also found in the luminal cells of the prostate cancer precursor lesion, PIN, indicating Cdc37 activation may be an important early step in prostate cancer development. To extend these findings, targeted Cdc37 overexpression in mouse prostate epithelium using the probasin promoter generated epithelial hyperplasia in the ventral prostate by 8 months of age (1). In mice Cdc37 has also been found to collaborate with c-myc in the development of tumors in multiple tissues suggesting it regulates a rate-limiting step in epithelial cell transformation (2). However, the effect of Cdc37 on the proliferation and survival of primary human epithelial cells, cells in which in vitro and in vivo transformation is rare, is unknown.

The Mₖ 50,000 product of the mammalian cdc37 gene is a co-chaperone that is absolutely required for Hsp90 substrate-specific folding activity (3–6). Hsp90 family members are molecular chaperones that provide maturation and folding to a number of client polypeptides in an ATP-dependent manner. Cdc37 binds immature protein kinases through interaction with its NH₂-terminal region (7) and links these to the Hsp90 COOH terminus (8). In addition to a physical role in targeting kinases to Hsp90 for activation, yeast Cdc37 also exhibits protein chaperone activity (4) opening the possibility that the mammalian homologs may also possess some folding activity. Biologically, Cdc37 is critical not only for kinase activation but also protein stability. Blocking Cdc37 function in immortalized cell lines using either a dominant negative Cdc37 mutant (7) or inhibiting Hsp90 activity with ansamycins, such as geldanamycin (9), results in both decreases in steady-state protein levels and in the activity of client kinases, such as Cdk4, Raf-1, v-Src, Akt, and the androgen receptor (7, 10–13). One Cdc37 client kinase, Cdk4, is a critical component of the cell cycle machinery. The decision to progress through the cell cycle is modulated by a series of signal transduction pathways acting on genes required for cell cycle progression. Cyclin D proteins (D1, D2, or D3) form an active kinase complex with Cdk4 or Cdk6 that phosphorylates pRB and functions in the G₁ to S phase transition (14). These positive proliferation signals are antagonized by p16 and other members of the cyclin-dependent inhibitor family INK4a, which compete with cyclin D for binding to Cdk4 and Cdk6, thereby preventing kinase activity (15). Overexpression of either cyclin D1 or replacement of wild-type Cdk4 with a Cdk4 mutant that cannot bind the CDK inhibitor p16, Cdk4 R24C, leads to elevated Cdk4 activity, induction of proliferation, and elevated tumor incidence in mice (16, 17). Furthermore, fibroblasts derived from Cdk4 R24C homozygous mice or p16 knockout mice are immortal in culture (17). Thus, regulation of Cdk4 activity is critical in the proliferation of normal cells and appears to play a role in cancer susceptibility at least in mouse models.

Another Cdc37 client kinase is Raf-1, a critical signaling molecule in the mitogen-activated protein kinase pathway that transmits information from the cell surface to the nucleus and cell cycle machinery. Signaling through Raf-1 is commonly initiated by the membrane-bound family of Ras GTPases after stimulation by peptide ligands and growth factors resulting in receptor tyrosine kinase activation (18, 19). In the GDP-bound state, Ras recruits Raf-1 to the membrane, a process that results in Raf-1 activation (20). Activated Raf-1 can then phosphorylate MEK1/2, which can then phosphorylate and activate the p42/p44 mitogen-activated protein kinases extracellular signal-regulated kinase 1/2 (21). Signaling through Raf-1 positively regulates proliferation likely via the stimulation of cyclin D1 expression (22). Paradoxically, overexpression of activated Raf-1 can also lead to growth arrest and differentiation in both primary and immortalized cells (23, 24). It is unclear if these proliferative or inhibitory signals are cell type specific or dependent on the immortalization status.

In the present study we examine the role of Cdc37 in the proliferation of genetically intact nonimmortalized human epithelial cells using a unique cell model (25). Consistent with mouse transgenic studies, Cdc37 overexpression in HPECs is sufficient to enhance proliferation. Furthermore, Cdc37 inhibition results in an apoptotic

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2 To whom requests for reprints should be addressed, at Department of Surgery, University of Wisconsin, 600 Highland Avenue, K6/530, Madison, WI 53792. Phone: (608) 265-2225; Fax: (608) 265-8133; E-mail: jarrard@surgery.wisc.edu.

3 The abbreviations used are: PIN, prostate intraepithelial neoplasia; HPEC, human prostate epithelial cell; BrDU, 5-bromo-2-deoxy-uridine; PBST, PBS plus 0.1% Tween 20; RT-PCR, reverse transcriptase-PCR; Cdk, cyclin-dependent kinase; ΔRaf/ER, Raf-1 estrogen receptor fusion construct; MEK, mitogen-activated protein kinase/extracellular signal-regulated protein kinase.
response suggesting a role for Cdc37 in cell survival. This study supports the hypothesis that Cdc37 overexpression in the prostate can positively regulate growth, inhibit cell death, and play an early role in the progression of human prostate cancer.

**MATERIALS AND METHODS**

**Primary Cell Culture.** Prostate tissue was obtained under an approved Institutional Review Board protocol from men (ages 44–66) undergoing cytoprostatectomy for bladder cancer at the University of Wisconsin Hospital and Clinics. Histology confirmed that no bladder or prostate cancer was present in the prostate tissue harvested for our studies. Prostate epithelial cultures were established as described previously (26). Prostate tissues were minced with a scalpel and digested in a solution containing collagenase (500 units/ml; Sigma, St. Louis, MO) and plated on collagen-coated plates. Cells were harvested, and processed using an anti-BrdU monoclonal primary antibody, and analyzed by fluorescence-activated cell sorting (Fig. 1).

**SA-β-Gal Staining.** Cells growing in collagen-coated p35 dishes were washed twice in 1 × PBS and fixed in a PBS-buffered solution of 2% paraformaldehyde/0.2% paraformaldehyde for 5 min. Cells were washed again in 1 × PBS and stained 16 h at 37°C in a solution as described (29).

**BrdU Labeling and Cell Cycle Analysis.** Cells were fed with fresh media 24 h before BrdU labeling. HPECs were BrdU labeled (Sigma) for 1 h, harvested, and processed using an anti-BrdU monoclonal primary antibody followed by a goat antimouse FITC-conjugated secondary according to the manufacturer’s directions (Becton-Dickinson Immunocytochemistry Systems, San Jose, CA). Cells were analyzed with a FACScan (Becton-Dickinson Immunocytochemistry Systems) and the percentage of BrdU-positive cells (10,000 gated events) was determined using CellQuest software (Becton-Dickinson Immunocytochemistry Systems). Cell cycle phase was determined using MODFIT software (Becton-Dickinson Immunocytochemistry Systems).

**Western Blot Analysis.** Western blots were performed as described (27) from three independent infections. Cells were harvested by trypsin-EDTA and washed in 1 × PBS. Protein was extracted by freeze thawing three times in ECB buffer (50 mM Tris (pH 8.0), 125 mM NaCl, 100 mM NaF, 0.5% NP40, 200 μM Na3VO4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 50 μg/ml phenylmethylsulfonyl fluoride). Protein extracts were quantified using the Bradford assay. Twenty-five μg of whole cell extract were separated on polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and blocked in 5% nonfat dry milk in PBS-T. Polyclonal antibodies to Cdk4 (C-22), Cdk6 (C-21), and Raf-1 (C-20), in addition to monoclonal antibodies to cyclin D1 (A-12) and Cdc37 (N-18), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody to p16INK4A (AB-1) was obtained from Calbiochem (La Jolla, CA). The monoclonal antibody to α-tubulin (AB-1) was obtained from Oncogene (Cambridge, MA). Monoclonal antibodies to phospho S217/221 MEK1/2 and MEK1/2 were obtained from Cell Signaling Technologies (Beverly, MA). Secondary goat antimum IgG (horseradish peroxidase conjugate) and goat antirabbit IgG (horseradish peroxidase conjugate) antibodies were obtained from Pierce (Rockford, IL). Antibodies were applied in 2.5% nonfat dry milk in PBS-T for 1 h and washed three times for 10 min in PBS-T. A loading control, α-tubulin immunoblotting was carried out on each filter. Bound antibody was detected using chemiluminescence (Pierce).

**RESULTS**

**Cdc37 Overexpression Increases BrdU Incorporation in HPECs.** Using a retroviral system, Cdc37 or the empty pBABE puro vector was expressed in primary HPEC cultures. Infected cells were selected with puromycin until no viable cells remained in the uninfected cultures (72 h). No morphological changes were apparent between Cdc37 overexpressing and control cells (data not shown). To determine the percentage of cells actively undergoing DNA synthesis, cells were labeled with the nucleotide analogue BrdU, a commonly used method to quantitate proliferation. At 8 days postinfection, HPECs were pulsed with BrdU for 1 h, labeled with an anti-BrdU antibody, and analyzed by fluorescence-activated cell sorting (Fig. 1). Cells overexpressing Cdc37 showed a consistent marked increase in the number of BrdU-positive cells compared with the pBABE vector-infected control cells (66–123% increase; Fig. 1A) and a decrease in the number of cells in G0/G1 (Fig. 1B).

**Cdc37 Overexpression Leads to Alterations in Raf-1 Activity and Modulates the Cdk4 Pathway.** We next surveyed for molecular changes that may lead to enhanced proliferation in Cdc37-overexpressing HPECs. Because Raf-1 and Cdk4 are known Cdc37 client kinases, we focused on genes involved in these pathways. Western blot analysis was performed on protein extracts isolated from HPECs overexpressing Cdc37 or the vector only (Fig. 1C). Cdc37 protein was overexpressed at high levels compared with endogenous levels. Raf-1 steady-state levels were unchanged. However, Raf-1 activity, measured by the abundance of phosphorylated S217/221 MEK1/2, a direct target of Raf-1 (30), was consistently elevated in Cdc37-overexpressing cells, whereas total MEK1/2 levels were unchanged. Increases in Cdk4 levels and markedly reduced p16 expression were also consistently observed in Cdc37-overexpressing HPECs. No changes in cyclin D1 or Cdk6 expression were detected. Cyclin D2 and D3 were not detectable in HPECs at the protein level. Thus, Cdc37-driven proliferation may be mediated through increased Raf-1 and/or Cdk4 activity.
Fig. 1. Cdc37 overexpression increases proliferation and alters expression of cell cycle-related genes. In A, primary HPECs from individual patients were retrovirally infected with a Cdc37 construct or vector only control, selected with puromycin, and analyzed for proliferation through monitoring BrdU incorporation at 8 days postinfection. The values reflect the percentage of cells staining positively with an anti-BrdU antibody indicating DNA synthesis. In B, cell cycle analysis of the infected HPEC cultures was modeled using MODFIT software. This analysis demonstrates a consistent decrease in the G0-G1 DNA content in cells overexpressing Cdc37 compared with the pBABE puro vector only cells. In C, cellular extracts were immunoblotted for known Cdc37 targets and genes involved in the Cdk4/6 pathway. Greater p-MEK1/2 and Cdk4 levels were consistently found in Cdc37-overexpressing protein extracts, as well as decreases in p16 expression. The p-MEK1/2 antibody detects phosphorylated S217 and S221 residues.

Blocking Cdc37 Function Leads to Growth Arrest and Apoptosis. To address whether loss of Cdc37 function inhibits proliferation in normal epithelial cells a dominant negative Cdc37 construct, Cdc37ΔC, was used. This protein retains the ability to bind protein kinases and homodimerize. However, it cannot associate with Hsp90 because of a COOH-terminal truncation; leading to a block in the maturation of Cdc37 client polypeptides (7). By 4 days postinfection, distinct morphological changes were readily apparent in HPECs expressing Cdc37ΔC when compared with control cells. The majority of mutant Cdc37-expressing cells lost their characteristic cuboidal epithelial morphology and became contracted and highly light refractile (Fig. 2A). Hoechst 33342-stained cells were analyzed by fluorescent microscopy to assess nuclear DNA integrity. An abundance of fragmented, or pyknotic, nuclei indicated the mechanism of cell death was apoptotic (Fig. 2B). By 8 days postinfection, only 10–25% of Cdc37ΔC-expressing cells remained attached (data not shown).

Cdc37ΔC and control pBABE puro-infected cultures were assayed for proliferation by BrdU incorporation at 5 days postinfection. Effective growth cessation (∼10-fold) was caused by Cdc37ΔC expression, with cells accumulating in G0-G1 (Fig. 2C). Existing antibodies recognizing epitopes in the COOH terminus were unable to detect the COOH-terminally truncated Cdc37. Therefore, RT-PCR was used to amplify a segment of the NH2 terminus to confirm Cdc37ΔC expression (Fig. 2D). Western analysis demonstrated that levels of the Cdc37 client kinases Raf-1 and Cdk4 were consistently reduced when compared with empty vector indicating the Cdc37ΔC-truncated protein was indeed preventing wild-type Cdc37 activity (Fig. 2D). In addition, endogenous Cdc37 expression was elevated in Cdc37ΔC-expressing cells, suggesting post-translational mechanisms can regulate Cdc37 expression (Fig. 2D). Thus, the Cdc37 dominant negative construct further demonstrates Cdc37 is critical for proliferation in HPECs and additionally plays a role in cell survival by preventing apoptosis.

Raf-1 Activation Causes Growth Arrest. Selective induction of specific kinase pathways activated by Cdc37 was then performed. To test if overexpressing Raf-1 alone could generate a proliferative response, an inducible activated ΔRaf:ER was stably incorporated into HPECs through retroviral infection. After infection, cells were drug selected and Raf-1 activity was induced with β-estradiol. After 6 days of Raf-1 induction cells acquired an enlarged, flattened morphology...
Fig. 3. Activated Raf-1 inhibits proliferation and leads to differentiation. HPECs were retrovirally infected with the \( \beta \)-estradiol inducible \( \Delta \)Raf-ER construct and selected with puromycin. In A, at 6 days postinduction, cells exhibited noticeable morphological alterations, becoming elongated with the presence of large vacuoles in many cells. In B, BrdU labeling revealed \( \beta \)-estradiol-induced growth arrest with a tendency toward a Gi-G1 block in \( \Delta \)Raf:ER-infected cells. The presence of \( \beta \)-estradiol on vector only infected cells (NP306 pLXSN) did not have an effect on cell growth. In C, molecular analysis shows S217/S221 MEK1/2, the downstream target of Raf-1, is hyperphosphorylated. Investigation into genes involved in modulating Cdk4 activity did not show a difference in cyclin D1, p16, or p21 levels, although Cdk4 expression was modestly decreased with activated Raf-1 overexpression.

Cdk4 Is Insufficient to Foster Proliferation. However, Cyclin D1 Overexpression Alone Is Sufficient to Generate a Proliferative Response. Molecular analysis of Cdc37-overexpressing HPECs suggests that the observed proliferative increases involve enhanced Cdk4 activity. Both Cdk4 protein increases and p16 repression may contribute to this increase. We manipulated HPECs by two methods to address the impact greater Cdk4 activity has on proliferation. First, HPECs were infected with Cdk4 or the pBABE puro control vector and selected with puromycin. Cdk4 was unable to reproducibly induce an increase in the BrdU proliferation index (Fig. 4A). However, consistently fewer cells accrued in G1 and a correspondingly higher Gi-M DNA content was found (Fig. 4B).

Given the difficulty of abrogating p16 expression in primary cell cultures, we used cyclin D1 as an additional molecular tool to determine whether elevated Cdk4 activity could induce proliferation in HPECs. Increasing levels of the Cdk4/6-binding partner cyclin D1 is an established mechanism for increasing Cdk4 activity (16, 32, 33). After drug selection, infected HPECs were assayed for proliferation by BrdU incorporation. Cyclin D1 generated a marked proliferation increase (51–94% higher; Fig. 5A) leading to both decreases in the Gi-M and Gi-G1 DNA content (Fig. 5B). Cellular extracts confirmed cyclin D1 overexpression in transfected cells (Fig. 5C). These experiments indicate that cyclin D1 generates a proliferative response similar to Cdc37 and Cdk4 activity is rate limiting in HPEC cell cycle progression.

DISCUSSION

It is generally accepted that cancer arises from a combination of uncontrolled proliferative cues, as well as inhibition of cell death pathways. As normal prostate cells transform into high-grade PIN lesions, a putative prostate cancer precursor, there is an increase in the number of cells proliferating (34). Progression to localized prostate
Cdc37 OVEREXPRESSION ENHANCES PROLIFERATION

We document for the first time that Cdc37 overexpression leads to a consistent, marked increase in the proliferation rate of normal HPECs. Our findings are consistent with the demonstration that targeted Cdc37 overexpression in the mouse prostate (PB-Cdc37.1 line) leads to the development of epithelial hyperplasia and dysplasia in >50% of prostatic acini (1). The probasin promoter fragment used in the transgenic mouse study is targeted to luminal epithelial cells. However, because secretory luminal cells are terminally differentiated, a proliferating luminal precursor cell may be induced (35). Our collagen-based model represents a proliferative population of epithelial cells that express both basal and luminal markers based on our previous work and others (26, 36, 37). This characterization is consistent with an amplifying or intermediate population of cells in vivo that express characteristics of both basal and luminal cells and has been proposed to represent a cell of origin for prostate cancer (38). Thus, Cdc37 induction in HPECs reproduces several aspects of human disease.

A second important observation was that the inhibition of Cdc37 function not only halted proliferation but also efficiently induced apoptosis. This result is not completely surprising as CDC37-null Saccharomyces cerevisiae are not viable (39). This finding raises the possibility that Cdc37 overexpression may also be antiapoptotic. A role for Cdc37 in the resistance to apoptosis is supported by recent reports demonstrating that Cdc37 binds to, stabilizes, and is required for maximal Akt activity (12). Because Akt is part of an important survival pathway, reduced Akt activity may sensitize primary HPECs to cell death. It is possible that both higher proliferation rates and apoptosis resistance underlie the hyperplastic growth seen in mouse prostate tissues overexpressing Cdc37 (PB-Cdc37.1) and in human prostate cancers (1). Additional studies will determine whether Cdc37 overexpression can also confer apoptosis resistance in vitro.

Our analysis of Cdc37-overexpressing HPECs demonstrated the activation of several growth control pathways, in conjunction with an increase in proliferation, including Raf-1. Raf-1 activity, measured by phosphorylation of its downstream target MEK1/2, was induced in Cdc37-overexpressing cells. Activated Raf-1 overexpression can induce a differential effect in various cell types. In a number of immortalized human and rodent cell lines, Raf-1 activation induces malignant progression (28). Conversely, activated Raf-1 uniformly induces growth arrest in finite life span human cells, although arrest also occurs in selected immortalized cell lines, including the prostate cancer cell line LNCaP (23, 24, 40). The mechanism behind Raf-1-induced growth arrest is unclear; however, it does not appear to require intact p53 or pRB pathways (23, 24). This differential response to overexpressed Raf-1 may be derived from genetic alterations that occur during the conversion process from a finite life span to immortalization. Strikingly consistent with previous studies in primary mammary cells, we found activated Raf-1 overexpression resulted in growth inhibition, similar morphological alterations, and a lack of senescence-associated β-galactosidase staining (24). The only cell cycle-related molecular alteration that we observed was a decrease in Cdk4 expression with Raf-1 induction. We do not, however, know what led to this repression or if it contributes to growth arrest. It is important to point out that phosphorylated MEK1/2 levels generated by overexpression of activated Raf-1 are significantly greater than levels derived by Cdc37 overexpression. We cannot rule out the possibility that modest, physiological Raf-1 activity elevations confer greater proliferation rates.

We found that Cdc37 overexpression induces Cdk4 expression and decreases p16 levels, strongly suggesting that an increase in Cdk4 kinase activity occurs. Unfortunately, because of low cell numbers after infection and selection, a limitation of working with mortal HPECs, we were unable to directly assay Cdk4 activity using kinase assays. To model increased Cdk4 activity, we overexpressed the regulatory Cdk4-binding partner, cyclin D1, which results in an increase in proliferation similar to that seen with Cdc37. Increasing levels of the Cdk4/6 binding partner cyclin D1 is an established mechanism for increasing Cdk4 activity (16, 32, 33). Overexpressing activated Cdk4 was not sufficient to confer cellular proliferation in HPECs, indicating that Cdk4 levels are not rate limiting, a finding noted in other primary cells (32). In addition, our finding that epithelial cells overexpressing Cdk4 arrest in G2 suggests they are able to partially bypass G1 but require additional factors to pass through G2. In sum, these data are consistent with the hypothesis that Cdc37 expression induces Cdk4 activity and that increased Cdk4 activity promotes proliferation in normal HPECs. However, as Cdc37 associates with other known and likely other uncharacterized client kinases, the manner through which Cdc37 promotes HPEC growth is likely multifactorial.

Because the Hsp90/Cdc37 association is important for both proliferation and survival, it is a potential target in cancer therapy (41). The drug 17-allylamino, 17-demethoxy-geldanamycin (17-A-GA) indirectly destabilizes kinases that cells need for survival by inhibiting Hsp90 and its related family members (9). Phase I clinical trials for the use of 17-A-GA are currently ongoing (42). However, selectivity between cancer cells and normal cells may be difficult because Hsp90 provides critical functions for a number of normal cellular processes.

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**Fig. 5.** Cyclin D1 overexpression is sufficient to drive proliferation. In A, HPECs retrovirally overexpressing cyclin D1 or the vector only were assayed at 8 days postinfection by BrdU analysis. B, the cell cycle profile determined by propidium iodide staining. Note fewer cells in both the G0–G1 and G2 phases in cells overexpressing cyclin D1. C, immunoblotting analysis demonstrating cyclin D1 overexpression.
A more direct and selective target may be Cdc37. Blocking Cdc37 co-chaperone activity would inhibit growth in overexpressing cells, such as prostate tumors, yet still allow Hsp90 to associate with other biologically important cochaperones, such as p23, HOP, and Hsp70. This Cdc37-specific strategy may result in reduced cytotoxicity compared with anti-Hsp90 drugs. The current findings in our prostate epithelial cell model suggest a strategy that inhibiting Cdc37 has therapeutic value.

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