

# Interleukin-8 Signaling Promotes Translational Regulation of Cyclin D in Androgen-Independent Prostate Cancer Cells

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## Abstract

We have shown previously that interleukin-8 (IL-8) and IL-8 receptor expression is elevated in tumor cells of human prostate biopsy tissue and correlates with increased cyclin D1 expression. Using PC3 and DU145 cell lines, we sought to determine whether IL-8 signaling regulated cyclin D1 expression in androgen-independent prostate cancer (AIPC) cells and to characterize the signaling pathways underpinning this response and that of IL-8-promoted proliferation. Administration of recombinant human IL-8 induced a rapid, time-dependent increase in cyclin D1 expression in AIPC cells, a response attenuated by the translation inhibitor cycloheximide but not by the RNA synthesis inhibitor, actinomycin D. Suppression of endogenous IL-8 signaling using neutralizing antibodies to IL-8 or its receptors also attenuated basal cyclin D1 expression in AIPC cells. Immunoblotting using phospho-specific antibodies confirmed that recombinant human IL-8 induced rapid time-dependent phosphorylation of Akt and the mammalian target of rapamycin substrate proteins, 4E-BP1 and ribosomal S6 kinase, resulting in a downstream phosphorylation of the ribosomal S6 protein (rS6). LY294002 and rapamycin each abrogated the IL-8-promoted phosphorylation of rS6 and attenuated the rate of AIPC cell proliferation. Our results indicate that IL-8 signaling (a) regulates cyclin D1 expression at the level of translation, (b) regulates the activation of proteins associated with the translation of capped and 5'-oligopyrimidine tract transcripts, and (c) activates signal transduction pathways underpinning

AIPC cell proliferation. This study provides a molecular basis to support the correlation of IL-8 expression with that of cyclin D1 in human prostate cancer and suggests a mechanism by which this chemokine promotes cell proliferation. (Mol Cancer Res 2007;5(7):737–48)

## Introduction

Elevated expression of the CXC chemokine, interleukin-8 (IL-8), has been correlated with the increased metastatic potential of melanoma (1), gastric (2), renal (3), ovarian (4-6), pancreatic (7-9), breast (10-13), and colorectal cancers (14, 15). Furthermore, IL-8 expression is associated with the disease progression of urogenital cancers, including transitional cell carcinoma of the bladder (16-19) and prostate cancer (20-24).

We previously showed elevated expression of IL-8 and each of its receptors, CXCR1 and CXCR2, in neoplastic cells of human prostate biopsy sections, suggesting that prostate cancer cells are subject to a continuous autocrine stimulus by this chemokine (25). The expression of IL-8, CXCR1, and CXCR2 in neoplastic prostate cells correlated with the expression of two markers of cell proliferation (cyclin D1 and Ki67) and microvessel density (CD34 staining). Furthermore, we showed both increased proliferation of the androgen-independent prostate cancer (AIPC) cell line PC3 in response to addition of exogenous IL-8 or inhibition of growth following antibody-mediated inhibition of endogenous IL-8 signaling in these cells.

An increased translation of proteins that regulate cell growth and cell cycle progression is fundamental to the increased proliferation rates of malignant tumor cells (26). Overexpression of the translation initiation factor eIF4E has been shown to alter cellular morphology, induce cell transformation, and potentiate cell proliferation, tumorigenesis, and metastasis, mediated through selective translation of mRNAs that encode oncoproteins involved in cell cycle progression (e.g., cyclin D1), cell survival (e.g., Akt), and angiogenesis (e.g., vascular endothelial growth factor and matrix metalloproteinase-9; ref. 27). Protein translation is controlled by the integration of numerous signals. Activation of the translational machinery has been shown in response to signals emanating from the Ras-, phosphatidylinositol 3-kinase (PI3K)-, mitogen-activated protein kinase (MAPK)-, and mammalian target of rapamycin (mTOR)-stimulated pathways (28). The latter of these signaling kinases, mTOR, is central to the regulation of protein translation through its concerted effects on the ribosomal

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**Note:** C.F. MacManus and J. Pettigrew contributed equally to this work.

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protein S6 kinases, the eukaryotic translation initiation factors (eIF), and the eukaryotic translation initiation factor binding proteins (4E-BP). The subsequent activation of the 48S ribosomal complex regulates the translation of mRNA transcripts with highly ordered 5'-oligopyrimidine tracts, whereas the activation of the eIF4F complex leads to translation of mRNA transcripts with highly structured, capped 5'-regions.

The D-type cyclins, D1, D2, and D3, play a critical role in regulating the commitment of cells to enter the cell cycle. Cyclin D associates with the cyclin-dependent kinases 4 and 6, forming protein complexes that underpin the progression from the early to mid-G<sub>1</sub> phase of the cell cycle. These cyclin D/cyclin-dependent kinase 4/6 complexes phosphorylate the retinoblastoma protein, inactivating its functional activity to complex with E2F and repress the activity of this transcription factor. The subsequent release of E2F leads to transcriptional induction of genes required for progression from G<sub>1</sub> to S phase, including that of cyclin E. Therefore, the expression of cyclin D1 is a key determinant of E2F-mediated transcriptional activity and cell proliferation. Consequently, cyclin D1 expression is tightly regulated to prevent unwarranted entry of cells into the cell cycle.

In this study, we show that IL-8 signaling regulates the translation of cyclin D1, potentiating its expression in cell-based models of AIPC. This observation is further supported by characterization of IL-8 signaling in AIPC cells confirming an activation of PI3K and mTOR signaling pathways and a phosphorylation-mediated downstream regulation of proteins implicated in cap-dependent and ribosome-mediated regulation of protein translation. Our observations enhance our understanding of signaling pathways modulated by IL-8 in AIPC cells, provide a molecular basis to explain our prior correlation of IL-8 expression with cyclin D1 expression in human prostate biopsy tissue, and suggest a mechanistic basis by which this chemokine regulates cell proliferation (25).

## Results

### *IL-8 Signaling Increases Cyclin D1 Expression in Prostate Cancer Cells*

Our prior studies on prostate biopsy tissue identified a statistically significant correlation between IL-8 and cyclin D1 expression in prostate cancer cells and showed that both endogenous IL-8 and exogenously administered recombinant human IL-8 (rh-IL-8) were linked to AIPC cell proliferation (25). The primary objective of this study was to provide evidence that IL-8 signaling regulates cyclin D1 expression in AIPC cells. Our current experiments were conducted on two AIPC cell lines, PC3 and DU145, each of which was shown to express CXCR1 and CXCR2 receptors by immunoprecipitation-immunoblotting (Fig. 1A) and flow cytometry analysis (data not shown), consistent with our prior characterization of their expression in epithelial-derived prostate cancer cells.

Immunoblotting experiments confirmed that stimulation of AIPC cells with rh-IL-8 resulted in an increased cyclin D1 expression. Increased expression of cyclin D1 in PC3 cells was detectable as early as 2 min after stimulation with 3 nmol/L rh-IL-8 and remained elevated out to 60 min (Fig. 1B, *left*). A transient increase in cyclin D1 expression was also detected

in DU145 cells following stimulation with 3 nmol/L rh-IL-8 (Fig. 1B, *right*). In addition to showing a time-dependent relationship, we confirmed the specificity of this response by further showing a concentration dependence to IL-8-promoted cyclin D1 expression, shown for PC3 cells in response to increasing concentrations of rh-IL-8 for 10 min (Fig. 1C, *left*). We also examined whether blockade of constitutive IL-8 signaling in PC3 cells, conducted by either targeting the constitutive IL-8 expression of IL-8 or inhibiting each of its receptors with neutralizing antibodies, would diminish the basal expression of cyclin D1 observed in PC3 cells. Administration of neutralizing antibodies against IL-8, CXCR1, and CXCR2 (at concentrations shown previously to block cell proliferation; ref. 25) each attenuated the basal expression of cyclin D1 detected in PC3 cells (Fig. 1C, *right*).

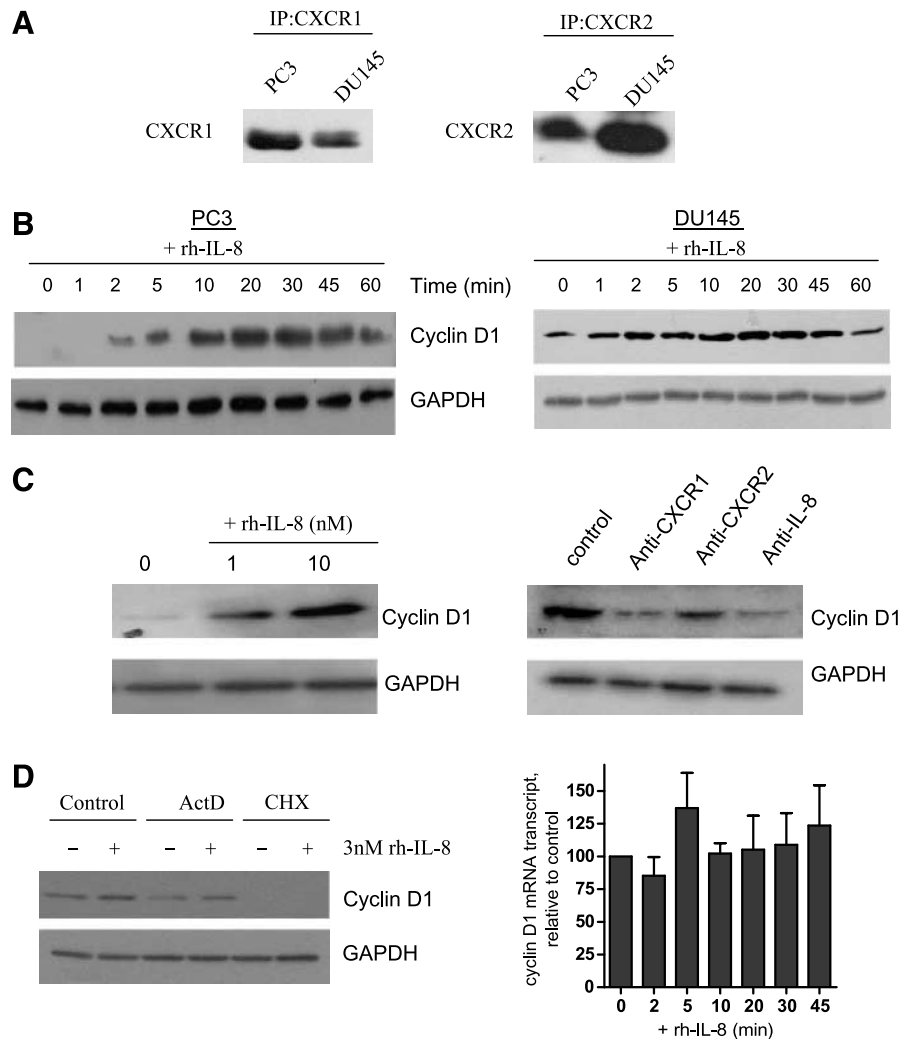
The rapidity with which IL-8 signaling potentiated cyclin D1 expression in AIPC cells suggested to us that this response was consistent with chemokine-promoted regulation of protein translation as opposed to transcriptional regulation of the *cyclin D1* gene. To test this hypothesis, AIPC cells were stimulated with 3 nmol/L rh-IL-8 in the absence or presence of either the RNA synthesis inhibitor actinomycin D or the translation inhibitor cycloheximide. Pretreatment with actinomycin D had no effect on the ability of rh-IL-8 signaling to transiently increase cyclin D1 expression in these cells (Fig. 1D, *left*). However, treatment with cycloheximide abolished both the basal expression and the IL-8-promoted increase in cyclin D1 expression in PC3 cells. Quantitative PCR also confirmed that rh-IL-8-induced signaling did not increase cyclin D1 mRNA transcript levels over the same time course in which we observed increased cyclin D1 protein expression (Fig. 1D, *right*).

### *IL-8 Signaling Promotes Activation of PI3K/Akt Signaling in AIPC Cells*

The secondary objective of this study was to identify the molecular basis by which IL-8 signaling may regulate cyclin D1 expression and promote cell proliferation. In a series of further immunoblotting experiments, we sought to show that IL-8 induced the activation of signaling pathways in AIPC cells known to be associated with the regulation of the translation machinery and/or regulation of cell proliferation.

Activation of the PI3K pathway in AIPC cells was initially studied using two antibodies that detect specific phosphorylation on the Thr<sup>308</sup> and Ser<sup>473</sup> residues of the PI3K substrate protein Akt. Phosphorylation of Thr<sup>308</sup> was detectable 2 min after stimulation with 1 nmol/L rh-IL-8 and remained elevated above basal levels for greater than 10 min in PC3 cells (Fig. 2A, *left, top*). We also detected prolonged phosphorylation of the Ser<sup>473</sup> residue of Akt in response to stimulation with 1 nmol/L rh-IL-8, being first detectable at 1 min, reaching a peak level at 10 min and returning to basal levels by 60 min (Fig. 2A, *left, middle*). Phosphorylation of Akt on Thr<sup>308</sup> and Ser<sup>473</sup> was also detected in DU145 cells in response to stimulation with rh-IL-8 (Fig. 2A, *right*). The magnitude of rh-IL-8-induced Ser<sup>473</sup> phosphorylation on Akt relative to Akt expression was further analyzed by densitometry (Fig. 2B, *left*), which revealed that the intensity and the maintenance of phosphorylation on this residue were not as prolonged in the DU145 cells as that

**FIGURE 1.** IL-8 signaling regulates cyclin D1 expression in AIPC cells. **A.** Characterization of CXCR1 (left) and CXCR2 expression (right) in PC3 and DU145 prostate cancer cells by immunoprecipitation (IP)-immunoblotting. **B.** Immunoblot showing a transient time-dependent increase in cyclin D1 expression in PC3 cells (left) and DU145 cells (right) following stimulation with 3 nmol/L rh-IL-8. Blots are representative of three independent experiments. **C.** Left, immunoblot showing the concentration-dependent increase in cyclin D1 expression in PC3 cells in response to stimulation with 1 and 10 nmol/L rh-IL-8 for 10 min. Right, immunoblot showing the effect of abrogating constitutive IL-8 signaling in PC3 cells on cyclin D1 expression. Cells were electroporated with anti-IL-8 (2 µg/mL), anti-CXCR1 (4 µg/mL), or anti-CXCR2 (4 µg/mL) antibodies or an IgG-negative control antibody. **D.** Left, effect of 1 µg/mL actinomycin D (*ActD*) or 1 µg/mL cycloheximide (*CHX*) on the IL-8-stimulated cyclin D1 expression in PC3 cells. Cells were treated with these inhibitors for 3 h before stimulation with 3 nmol/L rh-IL-8 for 20 min. Treatment with actinomycin D had no effect on the IL-8-promoted increase in cyclin D1 expression, whereas basal and IL-8-promoted expression of cyclin D1 was attenuated by treatment with cycloheximide. Blots (A-D) are representative of three experiments and equivalent loading of protein samples was verified on all blots by detection of GAPDH expression. Right, the relative cyclin D1 mRNA transcript levels in PC3 cells in response to stimulation with 3 nmol/L rh-IL-8 over a 45-min time course. Columns, mean values calculated by analysis of four independent experiments; bars, SE.



observed in PC3 cells. Therefore, we propose that the prolonged Akt phosphorylation response observed in the PC3 cells may reflect the absence of the phosphatase PTEN in these cells.

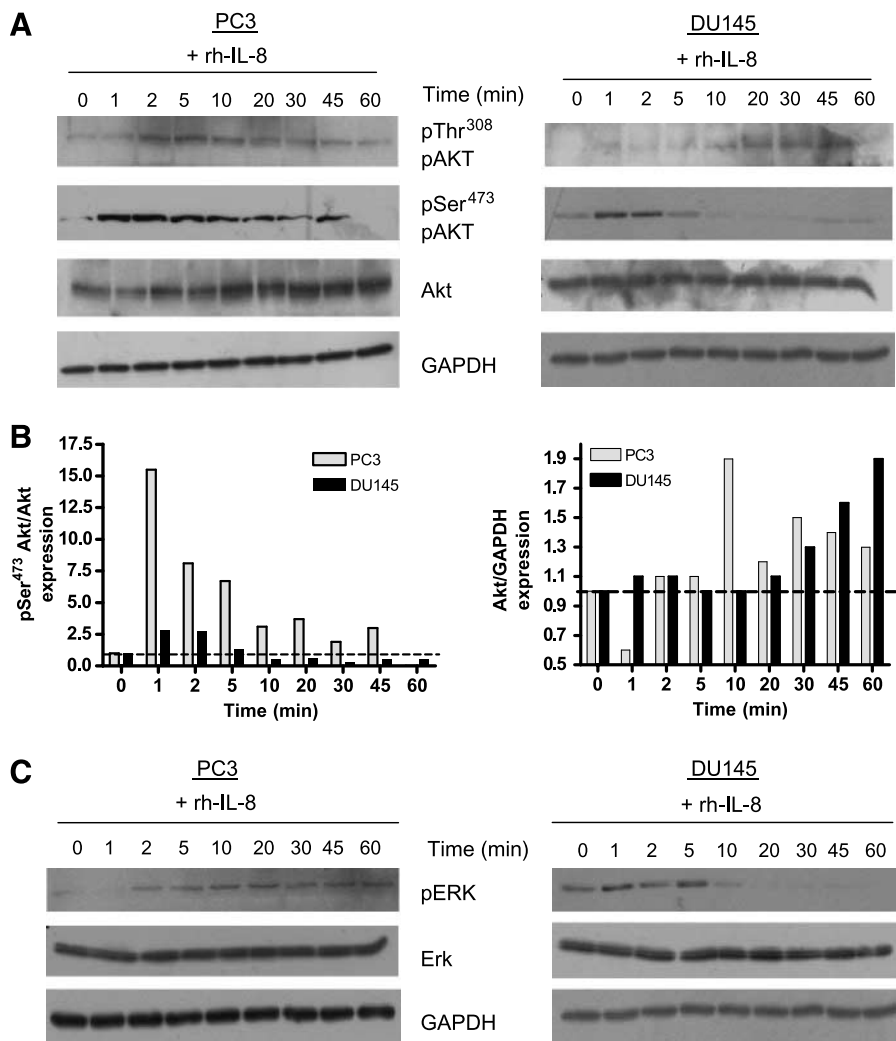
In addition to detecting increased phosphorylation of Akt, densitometry analysis of immunoblots also suggested that IL-8 signaling increased Akt expression in AIPC cells (Fig. 2B, right). This was particularly evident in PC3 cells where greater than 1.5-fold increases in Akt expression relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detectable 10 min after stimulation with rh-IL-8. Increases in Akt expression in DU145 cells were also indicated by densitometry analysis, detected at time points exceeding 30 min after stimulation with rh-IL-8 (Fig. 2B, right). Therefore, our experiments indicate that IL-8 signaling increases both the activation and the expression level of Akt in AIPC cells.

*IL-8 Signaling Promotes the Activation of Phospholipase D and Atypical Protein Kinase Cζ in AIPC Cells*

Phospholipase D (PLD) catalyzes the conversion of phosphatidylcholine to phosphatidic acid (PA) and is activated

by IL-8 signaling in neutrophils (29, 30). PA is an important upstream regulator of mTOR (31), atypical protein kinase Cζ (PKCζ; ref. 32), and PI3K activation (33). Addition of 3 nmol/L rh-IL-8 stimulated a rapid increase in PLD activity in PC3 cells, increasing basal PLD activity to a peak of 169 ± 26% (P < 0.05) of that detected in unstimulated cells within 1 min (Fig. 3A). Further experiments were conducted to determine the contribution of PA in mediating the IL-8-promoted increase in Akt signaling. Treatment of PC3 cells with 0.3% (v/v) butan-1-ol, an inhibitor of PLD activity, attenuated but did not abrogate the rh-IL-8-promoted phosphorylation of the Thr<sup>308</sup> residue of Akt (Fig. 3B).

Because PA is also a potent activator of PKCζ (32), immunoblotting experiments were conducted to determine if PKCζ was a downstream substrate of IL-8 signaling, examining the phosphorylation of PKCζ on residue Thr<sup>410</sup> in response to rh-IL-8 administration. Increased phosphorylation of PKCζ was detected in PC3 cells between 5 to 45 min after stimulation with rh-IL-8 (Fig. 3C, left, top). Further analysis of these immunoblots by densitometry confirmed that IL-8 signaling increased the phosphorylation of this kinase by a factor of



**FIGURE 2.** IL-8 signaling activates PI3K and MAPK signaling in AIPC cells. **A.** Immunoblots showing the time-dependent stimulation of PI3K activity in PC3 cells (*left*) and DU145 cells (*right*) in response to rh-IL-8 (1 nmol/L), indicated by increased phosphorylation of the substrate protein Akt on residues Thr<sup>308</sup> (*top*) and the Ser<sup>473</sup> residue (*middle top*). Immunoblotting was also conducted to analyze Akt expression in these samples (*middle bottom*). **B.** rh-IL-8 stimulated fold changes in the relative phospho-Akt (pAKT)/Akt ratio in PC3 and DU145 cells (*left*) and Akt expression detected in PC3 and DU145 cells (*right*), calculated by densitometry analysis of the respective immunoblots. **C.** Immunoblots showing the time-dependent phosphorylation of p42 Erk1 in PC3 cells (*left*) and DU145 cells (*right*) in response to 3 nmol/L rh-IL-8. Equal loading of protein in above experiments was confirmed by parallel analysis of the nonphosphorylated protein species in these samples and by reprobing the original membranes for GAPDH expression.

3.4-fold relative to either PKC (Fig. 3C, *right*) or GAPDH expression, whereas densitometry confirmed that the expression of PKC did not change relative to GAPDH expression in these cells in response to rh-IL-8 administration (data not shown). The rh-IL-8-promoted potentiation of PKC $\zeta$  phosphorylation could not be shown when cells had been pretreated with 0.3% (v/v) butan-1-ol, suggesting that PA is an intermediate promoting the IL-8-stimulated phosphorylation of this kinase (Fig. 3C, *left, middle top*).

#### IL-8 Signaling Underpins the Phosphorylation of the mTOR Substrate Protein 4E-BP1

PA and Akt are proposed to be independent activators of mTOR (27, 28, 31), suggesting that mTOR may be a downstream target of IL-8 signaling cascades in PC3 cells. To test this, we determined the effect of IL-8 signaling on the phosphorylation status of the downstream mTOR substrate, 4E-BP1. Consistent with the rapid increase in both PLD activity and Akt phosphorylation, we observed increased phosphorylation of 4E-BP1 in PC3 cells (Fig. 3D) within 5 min of rh-IL-8 stimulation, with peak phosphorylation occurring between 20

to 30 min after stimulation. Immunoblotting and densitometry analysis confirmed that this increase could not be explained by changes in 4E-BP1 expression. Similarly, IL-8 signaling was shown to increase 4E-BP1 phosphorylation between 20 to 45 min in DU145 cells without altering 4E-BP1 expression (data not shown). Therefore, this suggests that IL-8 signaling activates mTOR in AIPC cells, inducing downstream phosphorylation of the mTOR substrate 4E-BP1.

#### IL-8 Signaling Underpins the Phosphorylation and Activation of Ribosomal S6 Kinase

Extracellular signal-regulated kinase (Erk), PKC $\zeta$ , PDK1, and mTOR all modulate the selective phosphorylation of ribosomal S6 kinase (rS6K) at distinct serine and threonine residues (34-36). Because prior experiments suggested that each of these kinases is IL-8 signaling intermediate, we examined the effect of IL-8 signaling on the phosphorylation status of rS6K using immunoblotting. We detected a time-dependent phosphorylation of the Thr<sup>421</sup>/Ser<sup>424</sup> residues of both the cytoplasmic p70 and nuclear p85 S6K isoforms (upper and lower bands, respectively), in response to stimulation of PC3

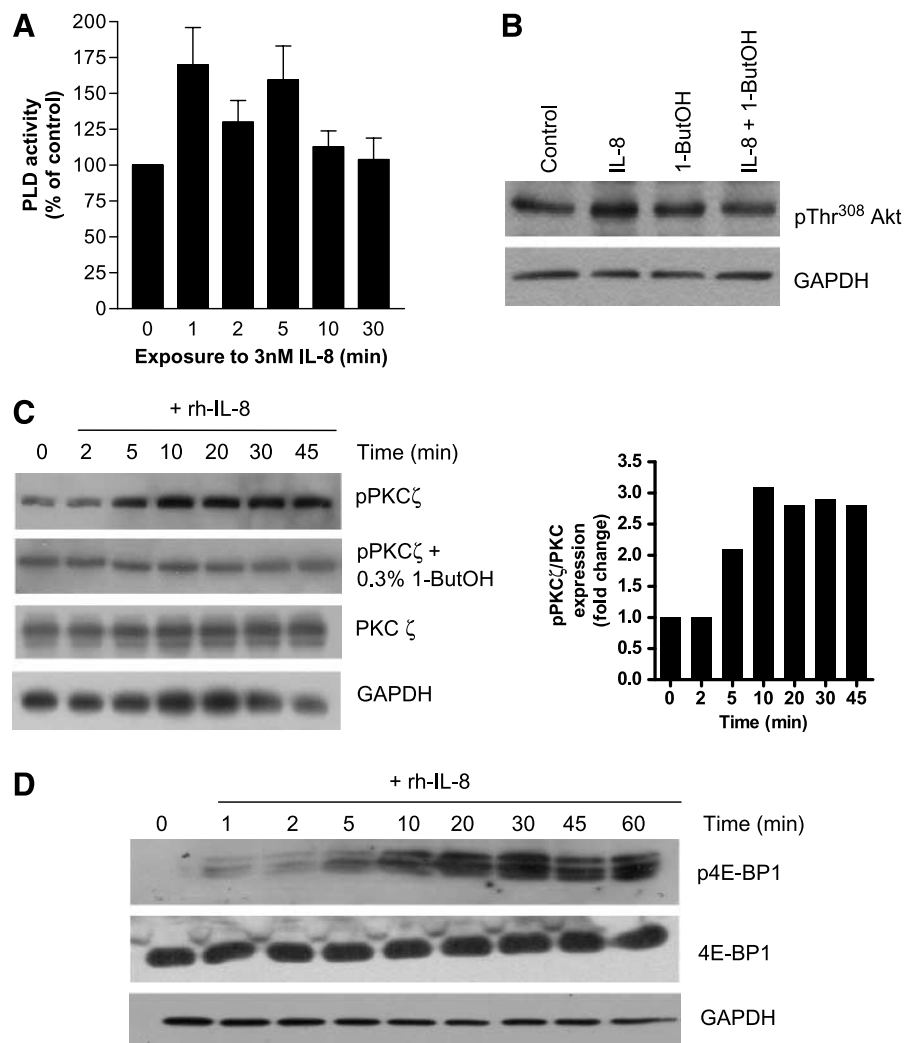
cells with 3 nmol/L rh-IL-8 (Fig. 4A, top). Phosphorylation of the Thr<sup>421</sup>/Ser<sup>424</sup> residues was clearly detectable out to 30 min after stimulation with rh-IL-8. Stimulation with rh-IL-8 promoted a biphasic phosphorylation of the Thr<sup>389</sup> residue of rS6K, with clearly distinguishable early and later peaks of phosphorylation (Fig. 4A, middle top). Although further experiments will be required to elucidate the mechanisms underpinning the early and late phosphorylation signals detected on this residue, it is possible that the initial response may be due to PA-initiated mTOR signaling, whereas the secondary response arises as a consequence of hierarchical phosphorylation promoted by Erk/PKC $\zeta$ -promoted phosphorylation of the Thr<sup>421</sup>/Ser<sup>424</sup> residues. In addition, elevated phosphorylation on the catalytically active Thr<sup>229</sup> residue of rS6K was detected 5 min after stimulation with rh-IL-8 and peaked between 10 and 20 min postchallenge. rS6K expression was unaffected by IL-8 signaling in PC3 cells.

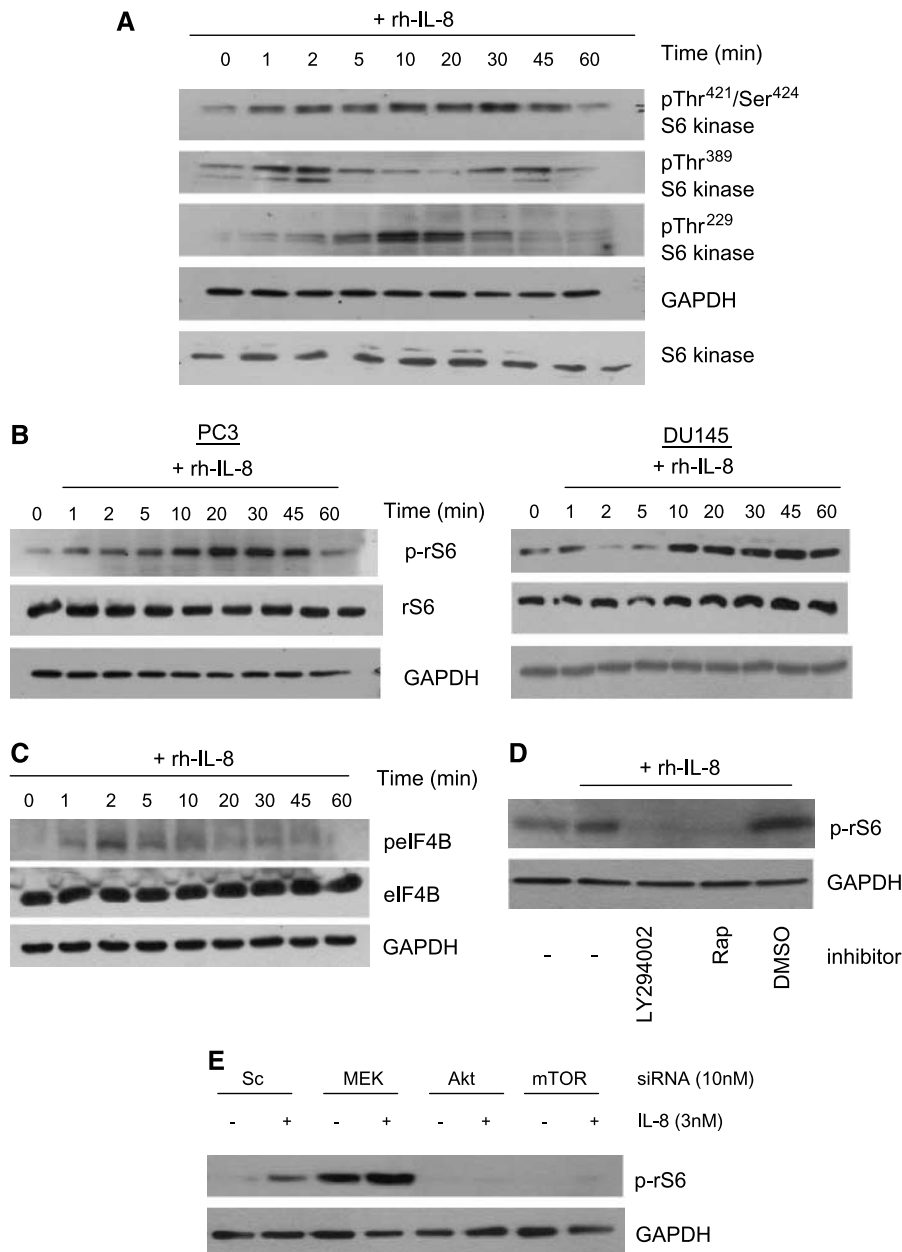
To determine whether IL-8 signaling increased S6K activity in AIPC cells, we examined the phosphorylation status of two substrate proteins, ribosomal S6 protein and eIF4B. Treatment of both PC3 cells (Fig. 4B, left) and DU145 cells (Fig. 4B, right) with 3 nmol/L rh-IL-8 induced a time-dependent

phosphorylation of ribosomal S6 protein, a response that was sustainable for 45 min in both cell lines. Consistent with the peak phosphorylation of the catalytically active Thr<sup>229</sup> residue of rS6K kinase following stimulation with rh-IL-8, the peak phosphorylation of ribosomal S6 protein was detected between 20 and 30 min after treatment with rh-IL-8. Although S6 protein expression was unchanged in the PC3 cell line, densitometry analysis of each replicate immunoblot confirmed that IL-8 signaling increased S6 protein expression in excess of 1.6- to 3.4-fold, 30 to 60 min after stimulation of DU145 cells (data not shown). Phosphorylation of eIF4B on the Ser<sup>422</sup> residue was detectable between 2 and 5 min in PC3 cells following stimulation with 3 nmol/L rh-IL-8 (Fig. 4C), in parallel with the early phosphorylation of the Thr<sup>389</sup> residue of rS6K.

In further experiments, administration of the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin attenuated IL-8-promoted phosphorylation of rS6 protein in PC3 cells (Fig. 4D). Suppression of Akt and mTOR expression using small interfering RNA (siRNA) also inhibited both the basal level and the 3 nmol/L rh-IL-8-promoted phosphorylation of

**FIGURE 3.** IL-8 signaling promotes the activation of PLD defining a pathway leading to the activation of PI3K, atypical PKC, and mTOR in AIPC cells. **A.** The activation of PLD activity in PC3 cells in response to the addition of 3 nmol/L rh-IL-8 was determined using colorimetric analysis of choline production. Columns, mean values determined in three individual experiments; bars, SE. **B.** Immunoblot showing that the rh-IL-8-promoted phosphorylation of the Thr<sup>308</sup> residue of Akt in PC3 cells is attenuated by pretreatment of the cells for 5 min with 0.3% (v/v) butan-1-ol (1-ButOH), an inhibitor of PLD. Where indicated, the cells were stimulated with 3 nmol/L rh-IL-8 for 2 min. **C.** Left, immunoblot showing a time-dependent phosphorylation of the atypical PKC $\zeta$  isoform in PC3 cells in response to stimulation with 3 nmol/L rh-IL-8 (top); immunoblot confirming that the IL-8-induced phosphorylation of PKC $\zeta$  in PC3 cells is attenuated by addition of 0.3% (v/v) butan-1-ol, suggesting that the response is mediated in part by PA (middle top). Equal protein loading was shown by reprobing for the nonphosphorylated species of PKC $\zeta$  (middle bottom) or GAPDH (bottom). Right, the rh-IL-8 stimulated fold change in the relative phospho-PKC $\zeta$  (pPKC $\zeta$ )/PKC $\zeta$  ratio in PC3 cells calculated by densitometry analysis of the respective immunoblots. **D.** Immunoblot showing a time-dependent phosphorylation of the mTOR substrate, the translation inhibitory protein 4E-BP1 in PC3 cells in response to stimulation with 3 nmol/L rh-IL-8. Each of the immunoblots shown in (B-D) is representative of three independent experiments.





**FIGURE 4.** IL-8 signaling promotes the phosphorylation and activation of ribosomal protein S6 kinase. **A.** Immunoblots showing time-dependent phosphorylation on three consensus phosphorylation sites of S6 kinase: Thr<sup>421</sup>/Ser<sup>424</sup> (top), Thr<sup>389</sup> (middle top), and Thr<sup>229</sup> (middle) residues on ribosomal protein (p70) S6 kinase (lower band) and p85 S6 kinase (upper band) in PC3 cells in response to stimulation with 3 nmol/L rh-IL-8. All residues are numbered according to the structure of the p70 isoform of this kinase. **B.** Immunoblot showing time-dependent phosphorylation of ribosomal S6 protein in PC3 (left) and DU145 cells (right) in response to 3 nmol/L rh-IL-8. **C.** Immunoblot showing time-dependent phosphorylation of Ser<sup>422</sup> of eIF4B in PC3 cells in response to stimulation with 3 nmol/L rh-IL-8. **D.** Effect of the PI3K inhibitor LY294002 (10  $\mu$ mol/L) and the mTOR inhibitor rapamycin (Rap; 10 nmol/L) on IL-8-induced phosphorylation of ribosomal S6 protein in PC3 cells. Cells were equilibrated with the signal transduction inhibitors for 3 h before experimentation and then stimulated for 20 min with 3 nmol/L rh-IL-8. **E.** Effect of attenuating Akt and mTOR expression using siRNA on the IL-8-induced phosphorylation of ribosomal S6 protein in PC3 cells. Cells were transfected with kinase-targeted or scrambled oligonucleotides for 72 h and then stimulated for 20 min with 3 nmol/L rh-IL-8. All blots in (A-E) are representative of two or three independent experiments. Equivalent loading of protein samples was verified on all blots by detection of the non-phosphorylated protein species and/or GAPDH expression.

rS6 protein in PC3 cells (Fig. 4E). In contrast, the inhibition of MAP/ERK kinase 1 (MEK1) expression using siRNA resulted in an increase in the phosphorylation of rS6 protein detected in both unstimulated and IL-8-stimulated cells.

#### Relationship of Constitutive IL-8, Akt, and mTOR to AIPC Cell Proliferation

Finally, experiments were conducted to show the relevance of endogenous IL-8 and each of the IL-8-promoted signaling pathways in regulating PC3 cell proliferation. An IL-8 siRNA strategy was adopted to deplete the endogenous levels of this CXC chemokine in these cells. Transfection of PC3 cells with 20 nmol/L of a commercial IL-8-targeted oligonucleotide pool reduced endogenous IL-8 secretion in PC3 cells from 40.7  $\pm$

4.3 to 9.8  $\pm$  1.4 ng/mL/10<sup>6</sup> cells/72 h, as measured by ELISA. Conversely, transfection with a scrambled oligonucleotide at this concentration had no significant effect on IL-8 synthesis or secretion. When studied using cell count assays, the suppression of endogenous IL-8 expression reduced the rate of PC3 cell proliferation by 23.2  $\pm$  6.8% relative to that observed in scrambled oligonucleotide-transfected cells over a 72-h period ( $P < 0.05$ ; Fig. 5A).

PC3 cells were treated with established signal transduction inhibitors to establish the relationship of each of these IL-8-induced signaling pathways to PC3 cell proliferation. The effects of these inhibitors on cell proliferation were determined by cell count analysis 72 h after treatment. Treatment of the PC3 cells with the MEK1 inhibitor U0126 reduced the detected

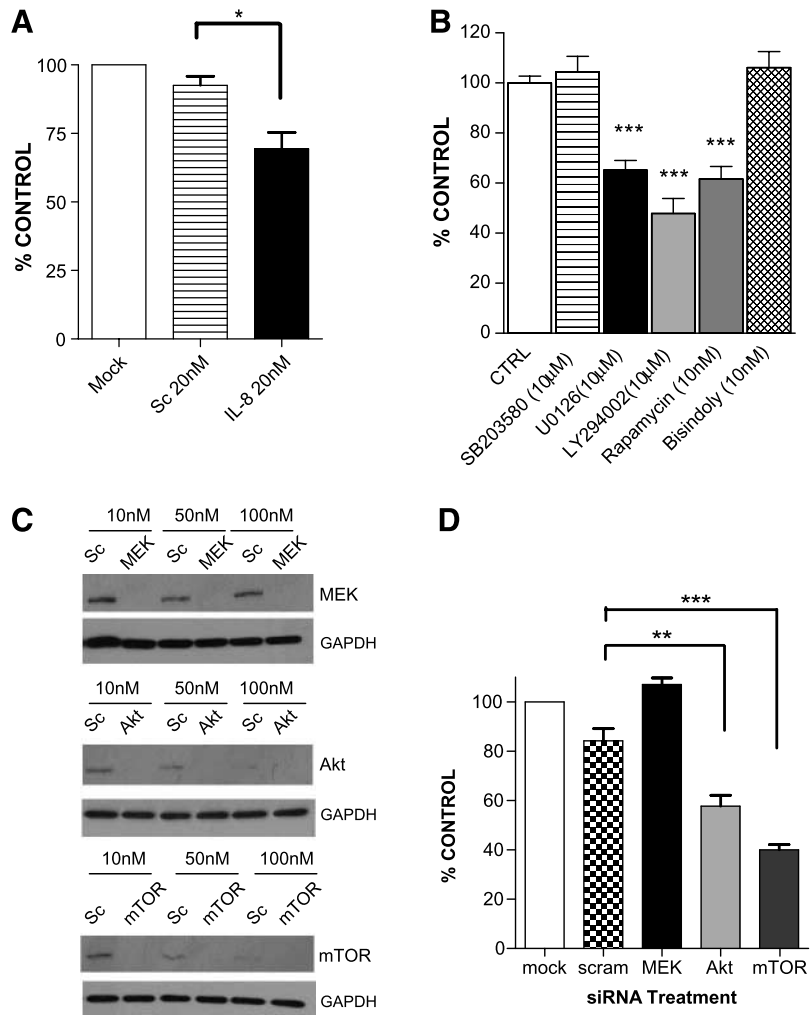
cell number to  $65.1 \pm 3.99\%$  of control ( $P < 0.001$ ). The PI3K inhibitor LY294002 decreased cell number to  $47.9 \pm 6.01\%$  ( $P < 0.001$ ) of control, whereas the mTOR inhibitor rapamycin reduced cell number to  $61.6 \pm 5.06\%$  of control ( $P < 0.001$ ; Fig. 5B). Treatment with the p38 MAPK inhibitor, SB203580, or the nonselective PKC inhibitor bisindolemaleimide had no demonstrable effect on PC3 cell proliferation. Parallel experiments confirmed that vehicle controls had no effect on cell proliferation at the concentrations used (data not shown).

To support the pharmacologic interventions, siRNA strategies were also used to examine the effect of attenuating Akt, Erk, and mTOR expression on the proliferation of PC3 cells. Specific protein knockdown was shown for each of the siRNA oligonucleotide pools by immunoblotting (Fig. 5C). Suppression of Akt expression reduced the detected cell number by  $26.5 \pm 6.7\%$  of that of the scrambled oligonucleotide control ( $P < 0.01$ ), whereas reducing mTOR expression decreased cell number by  $44.2 \pm 5.4\%$  of the scrambled oligonucleotide control ( $P < 0.001$ ; Fig. 5D). Surprisingly, the siRNA strategy to deplete MEK1 expression in PC3 cells failed to decrease the rate of cell proliferation, in marked contrast to the effect observed when cells were treated with U0126. Given the

discrepancy between the molecular and pharmacologic inhibition of MEK1, the cell proliferation assay was repeated using PD98059, a further inhibitor of MEK1. Administration of  $2 \mu\text{mol/L}$  PD98059 reduced cell number to  $63.8 \pm 7.7\%$  of control ( $P < 0.001$ ), comparable with the effect observed with U0126.

**Discussion**

We have previously reported elevated IL-8 and IL-8 receptor expression in human prostate cancer over that detected in normal prostate epithelium, the emergence of which was shown to correlate with markers of cell proliferation and microvessel density (25). Whereas angiogenesis has been proposed to contribute to IL-8-promoted tumor growth *in vivo* (21, 22), we have also shown a direct mitogenic effect of IL-8 on human prostate cancer cell lines *in vitro* (25). The aims of this current study were to provide evidence of a molecular link between IL-8 signaling and cyclin D1 expression and to further characterize the signaling pathways activated by IL-8 that underpin the mitogenic activity of this chemokine in prostate cancer cells.



**FIGURE 5.** Characterization of importance of endogenous IL-8 and multiple signaling pathways in underpinning AIPC cell proliferation. **A.** The suppression of endogenous IL-8 expression using a commercial siRNA strategy decreases the proliferation of PC3 cells. The effect of attenuating IL-8 expression using 20 nmol/L of an IL-8-targeted oligonucleotide pool is shown relative to the effect observed following transfection of PC3 cells with a scrambled oligonucleotide (Sc) at the same concentration or following mock transfection. Columns, mean value calculated from four independent experiments; bars, SE. **B.** The effect of various signal transduction pathway inhibitors, administered at indicated final concentrations, on the proliferation of PC3 cells was determined over the course of 72 h by cell count analysis. Columns, mean value calculated from four independent experiments; bars, SE. **C.** Immunoblots showing the siRNA-mediated depletion of MEK1, Akt, or mTOR expression in PC3 cells using 10, 50, or 100 nmol/L of the oligonucleotide pools. **D.** The effect on PC3 cell proliferation following siRNA-mediated depletion of MEK1, Akt, or mTOR expression in these cells. Data show the effects following transfection of PC3 cells with 10 nmol/L of the gene-targeted oligonucleotide pools or the scrambled oligonucleotide. Columns, mean value determined from four independent experiments; bars, SE. Statistical analysis of the data shown in (A), (B), and (D) was conducted using a Student's *t* test with significance indicated as follows: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

Immunoblotting experiments were conducted on representative cell-based models of androgen-independent disease where immunohistochemistry and *in situ* hybridization studies suggest that autocrine IL-8 signaling may have its most pronounced effect (25, 37). Administration of exogenous rh-IL-8 was shown to potentiate the expression of cyclin D1 in both PC3 and DU145 cells. Furthermore, the suppression of endogenous IL-8 signaling in PC3 cells using a series of ligand and receptor-targeted antibodies was accompanied by a reduction in the basal expression of cyclin D1. The promotion of cyclin D1 expression by IL-8 signaling showed both a time dependence and a concentration dependence, adding further weight to the specificity of the response. The increase in cyclin D1 expression was observed to be rapid in both cells and was apparently inconsistent with a transcriptional regulation of the cyclin D1 gene because no detectable increase in cyclin D1 mRNA transcript levels could be detected using quantitative PCR analysis over the same time course. Furthermore, administration of the RNA synthesis inhibitor actinomycin D failed to attenuate this rapid rh-IL-8-stimulated increase in cyclin D1 expression, suggesting that the response is consistent with translational regulation of cyclin D1.

Our observation that IL-8 signaling promotes a translation-mediated increase in cyclin D1 expression in AIPC cells *in vitro* characterizes a molecular mechanism to explain our prior correlation of IL-8 expression with that of cyclin D1 in the tumor cells of human prostate biopsy tissue. Furthermore, the demonstration that IL-8 signaling increases cyclin D1 expression is consistent with the reported mitogenic function of this chemokine in prostate (25), ovarian (5), pancreatic (7), and colorectal cancer cell lines (15). Cyclin D1 expression in prostate cancer is related to several malignant cellular features, including tumor metastasis classification, histologic differentiation, perineural invasion, DNA ploidy, S-phase fraction, expression of Ki67, and mitotic index (38) and bone metastasis (39). Therefore, our *in vitro* observations, indicating that IL-8 signaling regulates the expression of the cyclin D1 oncogene, provides further insights about the importance of this CXC chemokine in modulating the tumorigenicity and metastasis of prostate cancer (20-22).

Our experiments also establish that IL-8 signaling regulates pathways central to the regulation of protein translation. Specifically, mTOR plays an important role in integrating the activation of the translational machinery through a combination of phosphorylation-mediated activation of stimulatory proteins and inhibition of negative regulators (27, 28). Immunoblotting experiments using phospho-specific antibodies show that IL-8 signaling promotes the phosphorylation of two characterized substrates of mTOR (i.e., 4E-BP1 and rS6K). In addition, we showed that IL-8 signaling induced PA accumulation and promoted the phosphorylation of Akt, in AIPC cells, each of which is upstream activator of mTOR. Treatment with rapamycin and LY294002, inhibitors of the PI3K/Akt and mTOR kinase activity, respectively, attenuated the IL-8-promoted phosphorylation of rS6K, showing the importance of these signaling pathways to the IL-8-promoted regulation of the translational machinery. Therefore, we propose that IL-8 signaling underpins mTOR activation in AIPC cells, a

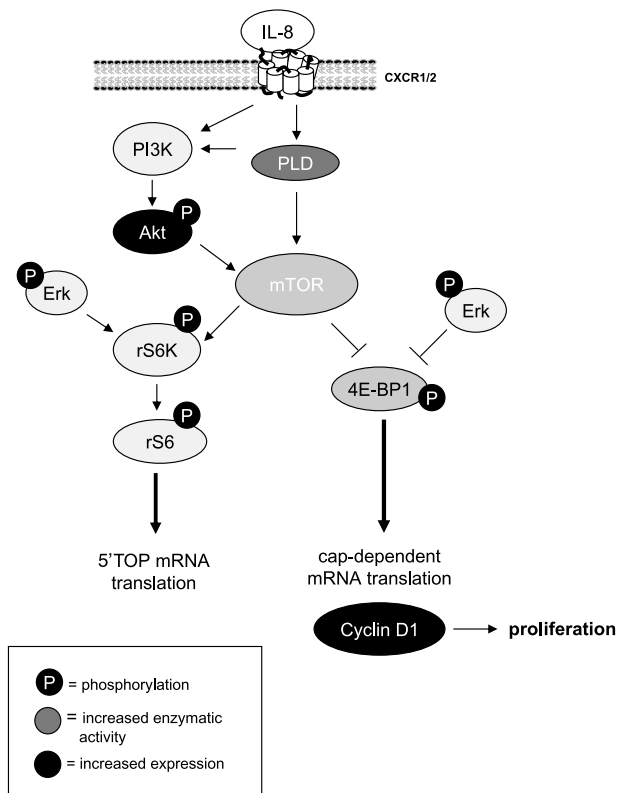
response that is likely mediated via both PLD-dependent and PI3K/Akt-dependent signaling.

The effects of mTOR activation in response to promotion of IL-8 signaling in AIPC cells resulted in concurrent phosphorylation of the translation inhibitory protein 4E-BP1 and Thr<sup>389</sup> of p70 rS6K. The phosphorylation of 4E-BP1 decreases the avidity of this protein to bind and sequester its substrate, the eIF4E protein, permitting eIF4E to interact with additional substrates to form the eIF4F complex and promote cap-dependent translation. With regard to rS6K, immunoblotting experiments confirmed that IL-8 signaling promotes phosphorylation of each of the serine and threonine residues implicated in the hierarchical sequence of ordered kinase-dependent activation of this protein (35, 36, 40), including the phosphorylation of Thr<sup>229</sup> residue, which is proposed to unleash the full catalytic activity to the kinase. Consistent with this, IL-8 signaling also promoted the phosphorylation of two downstream substrates of rS6K, the ribosomal S6 protein (rS6) and eIF4B (41). In each of our immunoblotting experiments examining the phosphorylation status of proteins associated with protein translation, the temporal relationship of these phosphorylation responses is consistent with (a) the identity of the upstream substrates (i.e., Erk, Akt, and mTOR) as known effectors of IL-8 signaling, (b) the kinetics with which IL-8 activates these upstream stimuli, and (c) the observation that the phosphorylation of the downstream substrates, rS6 and eIF4B, is detected in parallel with phosphorylation of the Thr<sup>229</sup> residue of the S6 kinase.

Therefore, our data suggest that IL-8 signaling regulates the assembly of both the 48S ribosomal protein complex and the eIF4F complex, each of which binds mRNA transcripts for translation (Fig. 6). In particular, our demonstration that IL-8 signaling promotes the phosphorylation of 4E-BP1 is consistent with the promotion of cap-dependent translation of cyclin D, providing a molecular basis to support our initial indication that IL-8 signaling regulates the translation of this oncogene in AIPC cells.

The final objective of the article was to show the relevance of IL-8-induced signaling pathways to the promotion of AIPC cell proliferation. siRNA-mediated suppression of endogenous IL-8 expression retarded PC3 cell proliferation, by a level consistent with that previously observed in these cells following the administration of a neutralizing antibody to this chemokine (25). Subsequently, siRNA-mediated depletion of mTOR and Akt expression and/or pharmacologic inhibition of mTOR and PI3K/Akt signaling using rapamycin and LY294002 identified the importance of these signaling pathways to AIPC cell proliferation. As reported previously in studies conducted in tumor cells and neutrophils (5, 42, 43), IL-8 signaling was also shown to induce the activation of MAPK signaling pathway in PC3 and DU145 cells, exemplified by time-dependent Erk phosphorylation. Pharmacologic inhibition of this pathway using each of the MEK1 inhibitors U0126 and PD98059 attenuated AIPC cell proliferation, suggesting that activation of MAPK signaling may also underpin the IL-8-promoted proliferation of AIPC cells, in a manner similar to that reported previously in ovarian cancer cells (5). Interestingly, siRNA-mediated inhibition of MEK1 failed to reduce the cell proliferation rate of these cells





**FIGURE 6.** Schematic summary of IL-8–promoted signal transduction pathways and the link to mRNA translation in AIPC cells. Proteins shown in this study to be phosphorylated or activated and/or whose expression was increased in response to IL-8 signaling in AIPC cells are shown.

and also resulted in an increased level of rS6 phosphorylation detected in these cells, suggesting that the oligonucleotide pool used to target this enzyme may have significant off-target and nonspecific effects that may invalidate its use in these cells.

As suggested by our prior study on human prostate biopsy tissue, the coexpression of IL-8 and IL-8 receptors on prostate cancer cells indicates that these cells are subject to a continuous autocrine/paracrine stimulus (25). Our current characterization of the signaling pathways activated by this chemokine in AIPC cells provides further insights into the relevance and importance of IL-8 in promoting the progression of prostate cancer. First, we have shown that IL-8 signaling increases both the expression and the activation of Akt in AIPC cells. Specifically, IL-8 signaling resulted in increased phosphorylation of both the Thr<sup>308</sup> and Ser<sup>473</sup> residues of Akt in PC3 and DU145 cells, although the response was more prolonged in the PC3 cells, most likely due to the absence of the endogenous phosphatase PTEN in these cells. The IL-8–induced phosphorylation of the Thr<sup>308</sup> residue also seemed to lag that of the phosphorylation on Ser<sup>473</sup> in either cell line, being most noticeable in the DU145 cells. Further detailed experiments will be required to map and characterize the pathways underpinning IL-8–induced activation of the PI3K/Akt pathway in each of these AIPC cells to explain the different kinetics of this response,

focusing on the receptor selectivity, G proteins, and the various primary effectors responsible for inducing the phosphorylation of this kinase. In addition, the prolonged phosphorylation of Thr<sup>308</sup> detected in the DU145 cells may result from cross-talk with other IL-8–induced signaling pathways. That said, the biological importance of PI3K/Akt signaling to human prostate cancer initiation and progression is increasingly acknowledged. Increased Akt phosphorylation has been detected using immunohistochemical and protein microarray studies on primary prostate tumors (44, 45). Other studies report that the progressive transition from histologically normal epithelium to prostate intraepithelial neoplasia is associated with increasing phosphorylation of Akt (46), whereas the induction of Akt activity in prostate cancer cells promotes androgen-independent growth and survival of prostate cancer cells (47). Furthermore, Akt1 deficiency dramatically inhibits prostate neoplasia development in PTEN<sup>+/-</sup> mice (48). Consistent with our current results, the activation of Akt has been shown to potentiate cyclin D1 expression in xenograft prostate cancer tissue (47), whereas *in vitro* studies have reported that pharmacologic inhibition of PI3K/Akt activity by LY294002 promotes G(1) cell cycle arrest and decreased expression of G(1)-associated proteins, including cyclin D1 and cyclin-dependent kinase 4 in prostate cancer cells (49). Second, we have shown that IL-8 signaling induces mTOR activity, a kinase identified as an important therapeutic target in prostate cancer. mTOR has been shown to mediate Akt-promoted G<sub>1</sub>-S cell cycle progression (49), whereas the inhibition of mTOR activity (a) attenuates the repopulation of surviving prostate cancer cells following chemotherapy in prostate cancer xenograft models (50), (b) sensitizes PTEN-deficient prostate cancer cells to chemotherapy (51) and radiotherapy (52), and (c) reverses the Akt-promoted prostate intraepithelial neoplasia phenotype in mice (53).

In summary, we report that IL-8 regulates cyclin D1 expression at the level of protein translation in AIPC cells, substantiating our prior correlation of IL-8 with cyclin D1 expression in human prostate biopsy tissue. Second, we provide evidence that IL-8 signaling in AIPC cells induces the activation of key regulators of both cap-dependent mRNA translation and 5′-oligopyrimidine tract mRNA translation. Finally, our experiments confirm that IL-8 signaling is coupled to the activation of PI3K/Akt/mTOR and MAPK signaling pathways that regulate the proliferation of these AIPC cells.

## Materials and Methods

### Chemicals and Reagents

LY294002 and rapamycin were obtained from Cell Signaling Technology. rh-IL-8 was sourced from PeproTech. All other chemicals were sourced from the Sigma Chemical Co. unless otherwise stated.

### Cells and Cell Culture

PC3 and DU145 human prostate adenocarcinoma cells were purchased from the American Type Culture Collection and cultured in RPMI 1640 and DMEM, respectively, supplemented

with 10% fetal bovine serum and 2 mmol/L L-glutamine (Invitrogen Life Technologies) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

#### Immunoprecipitation-Western Blotting

Cells were lysed in radioimmunoprecipitation assay buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% (w/v) Triton X-100, 0.1% SDS, 1 protease inhibitor tablet/10 mL (Roche Diagnostics, Inc.)], and the lysates were cleared by centrifugation at 15,000 × *g* for 20 min at 4°C. The supernatant fraction containing 0.1 or 1.0 mg protein was added to protein A-Sepharose beads carrying 20 μL anti-CXCR1 rabbit polyclonal (Serotec) or anti-CXCR2 rabbit polyclonal antibody (Santa Cruz Biotechnology Ltd.), respectively. Immunoprecipitation was done for 18 h at 4°C, following which the immunoprecipitated fraction was washed thrice with radioimmunoprecipitation assay buffer. For immunoblotting, protein samples retained on protein A-Sepharose beads were boiled in Laemmli's SDS sample buffer, resolved by SDS-PAGE, and electroblotted onto Hybond membranes (Hybond-P, Amersham Pharmacia). Membranes were probed with the following primary antibodies: (a) 1:500 dilution of anti-CXCR1 mouse monoclonal (Biosource) or (b) 1:500 dilution of anti-CXCR2 mouse monoclonal (Biosource) and subsequently with a 1:2,000 dilution of horseradish peroxidase-linked antirabbit IgG as the secondary antibody (Amersham Pharmacia). Antibody staining was detected using a chemiluminescence detection system (SuperSignal, Pierce).

#### Cell Count Proliferation Assay

Cells were seeded in 24-well plates at a density of 3 × 10<sup>4</sup> cells per well and allowed to adhere overnight. Cells were replenished by the addition of 1 mL fresh serum-free RPMI 1640 to each well, following which the signal transduction inhibitors were added. The cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere during experimentation. Cells were trypsinized and counted in triplicate using a Coulter Z Series particle count and size analyzer (Beckman Coulter). Cell numbers were normalized to control values and statistical analysis of the data was done using GraphPad Prism 3.0 software.

#### Immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer containing 100 nmol/L Na<sub>3</sub>VO<sub>4</sub>, and protein lysates were subjected to SDS-PAGE as described above. Hybond-P membranes were probed with the following primary antibodies sourced from Cell Signaling Technology at a dilution of 1:1,000 unless otherwise indicated: anti-phospho-Thr<sup>202</sup>/Tyr<sup>204</sup> p44/p42 Erk kinase, anti-p44/p42 Erk kinase, anti-phospho-Thr<sup>308</sup>-Akt, anti-phospho-Ser<sup>473</sup>-Akt, anti-Akt, anti-phospho-Thr<sup>37</sup>/Thr<sup>46</sup>-4E-BP1, anti-4E-BP1, anti-phospho-Thr<sup>421</sup>/Ser<sup>424</sup>-S6 kinase, anti-phospho-Thr<sup>389</sup>-S6 kinase, anti-phospho-Thr<sup>229</sup>-S6 kinase, anti-S6 kinase, anti-phospho-Ser<sup>235</sup>/Ser<sup>236</sup>-ribosomal S6 protein, anti-S6 protein, anti-phospho-Ser<sup>422</sup> eIF4B, anti-phospho-Thr<sup>403</sup>/Thr<sup>410</sup>-PKCζ/λ, anti-PKCζ/λ, and mouse monoclonal anti-cyclin D1 (1:500 dilution; Oncogene Research Products). Antibody staining was detected using the SuperSignal chemiluminescence detection system.

#### PLD Assay

Cells were initially seeded at a density of 5 × 10<sup>5</sup> cells per well in RPMI 1640, supplemented with 10% FCS and 20 mmol/L L-glutamine. The medium was replaced with serum-free RPMI 1640 and the cells rested for a further 24 h at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. rh-IL-8 (3 nmol/L) was added to cells for predetermined times (1, 2, 5, 10, or 30 min) and the reactions terminated by aspiration of the medium and two cell washes in ice-cold PBS. Cells were harvested by scraping in radioimmunoprecipitation assay buffer (250 μL) and were immediately snap frozen in liquid nitrogen. On thawing, cell debris was pelleted by centrifugation at 13,000 rpm for 5 min at 4°C and the supernatant was decanted for experimentation. PLD activity was measured using the Amplex Red PLD assay kit (Molecular Probes, Inc.) according to the manufacturer's protocol. The resulting fluorescence was detected using a fluorescence microplate reader at an excitation wavelength of 563 nm and an emission wavelength of 587 nm (Applied Biosystems Cytofluor 4000).

#### Real-time PCR Analysis

RNA was harvested from cultured cells using RNASat60 and cDNA was synthesized from 2 μg total RNA as per manufacturer's instructions (Invitrogen Life Technologies). Quantitative real-time PCR analysis was done on the DNA Engine Opticon 2 continuous fluorescence detector (MJ Research) with product amplification determined by SYBR Green 1 fluorescence detection (Finnzymes). The forward and reverse primers are as shown: cyclin D1, 5'-TTGCATGTTTCGTGGCCTCTA-3' (forward) and 5'-AAATCGTGCGGGTCTAA-3' (reverse), and 18S, 5'-CATTTCGTATTGCGCCGCTA-3' (forward) and 5'-CGACGGTATCTGATCGTC-3' (reverse). Standard cycling procedures were used with an annealing temperature of 55°C for all primer pairs tested. Specific amplicon formation with each primer pair was confirmed by melt curve analysis. Gene expression was quantified relative to an 18S housekeeping gene.

#### siRNA Transfection

Cells were seeded into either 24-well plates for cell count analysis or p90 tissue culture dishes for Western blot analysis at densities of 3 × 10<sup>4</sup> and 2 × 10<sup>5</sup> cells per well, respectively, and allowed to attach overnight. Akt1, MAPK kinase, FRAP-1 SMARTpool siRNA, and siCONTROL nontargeting siRNA 1 were obtained from Dharmacon. PC3 cells were transfected with 10, 50, or 100 nmol/L of the oligonucleotides using the Dharmafect 2 transfection reagent (Dharmacon) in accordance with the manufacturer's instructions. For investigation of IL-8 inhibition, IL-8 SMARTpool siRNA (Dharmacon) and siCONTROL nontargeting siRNA 1 were used at a concentration of 20 nmol/L. For quantification of protein knockdown, protein lysates were prepared as described previously and Western blot analysis was done 72 h after transfection to validate efficiency of the Akt1, MAPK kinase, and mTOR siRNA. Inhibition of *IL-8* gene function by IL-8-targeted siRNA was assessed by specific ELISA, as described previously (25).

### Data Analysis

Differences observed in functional assays between control and treated groups were analyzed for statistical significance using a two-tailed Student's *t* test comparison (GraphPad Prism).

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