Stabilization of cyclin D1 mRNA via the phosphatidylinositol 3-kinase pathway in MCF-7 human breast cancer cells

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Abstract

Treatment of quiescent MCF-7 human breast cancer cells with either the polypeptide growth factors insulin-like growth factor-I (IGF-I) or epidermal growth factor (EGF), the steroid hormone estradiol (E2) or the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) results in increased steady-state levels of cyclin D1 mRNA and protein. Unexpectedly, this elevation of cyclin D1 expression by all of these agents is inhibited by the specific phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002. Since transcriptional activation of the cyclin D1 promoter by EGF, E2 and TPA is independent of

PI3-K activity, these findings suggest a post-transcriptional role for PI3-K in the regulation of cyclin D1 expression. Here we show that inhibition of PI3-K by LY294002 decreases the half-life of the 4.5 kb cyclin D1 mRNA species. In contrast, the stability of the 1.5 kb cyclin D1 mRNA is not affected by PI3-K inhibition. PI3-K-mediated stabilization of mRNA is not a general phenomenon, since other rapidly regulated and unstable mRNAs, such as those encoding *c-fos*, *c-jun* and *c-myc*, are not stabilized upon activation of the PI3-K signaling pathway.

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Introduction

Orderly progression through the eukaryotic cell cycle is regulated by the activity of protein complexes consisting of a regulatory subunit called cyclin and a catalytic subunit, the cyclin-dependent kinase (cdk). Expression of the different cyclins is strictly orchestrated, so that each cyclin is only present during a specific time frame of the cell cycle (Nigg 1995). The first cyclins to appear when quiescent cells re-enter the cycle after growth-factor stimulation are the D-type cyclins (D1, D2 and D3). These cyclins bind to, and thereby activate, cdk4 and cdk6, which then phosphorylate the retinoblastoma protein (pRb). This phosphorylation leads to release of gene E2 promoter-specific factor (E2F) transcription factors from pRb, and in transcriptional activation of genes, containing E2F-sensitive promoters (Müller 1995, Weinberg 1995, Sanchez & Dynlacht 1996). Overexpression of cyclin D1, caused by genomic amplification, chromosomal translocation, increased mRNA stability or enhanced protein half-life, has been shown to be a feature of several human cancers, such as leukemias, breast tumors and sarcomas (Withers *et al.* 1991, Seto *et al.* 1992, Lebowitz *et al.* 1994, Welcker *et al.* 1996, Hosokawa *et al.* 1998). On the other hand, inhibition of cyclin D1 expression by microinjection of antibodies or antisense probes increases the length of the

G1 phase and inhibits proliferation (Filmus *et al.* 1994, Liu *et al.* 1995). These data establish cyclin D1 as an important regulator of G1 phase progression. The MCF-7 cell line is a human epithelial breast cancer cell line with the characteristic that serum-starved cells enter the cell cycle upon the sole addition of insulin-like growth factor-I (IGF-I) (van der Burg *et al.* 1988, Kary & Sirbasku 1988). Stimulation with IGF-I leads to enhanced expression of cyclin D1, hyperphosphorylation of pRb, DNA synthesis and cell division (Dufourny *et al.* 1997). Since upregulation of cyclin D1 is the first event in this process, and overexpression of cyclin D1 has been shown to be sufficient for growth of MCF-7 cells under low-serum conditions (Zwijsen *et al.* 1996), the increase in cyclin D1 levels seems to be the essential step in mitogenic IGF-I signaling. The signaling pathways that are activated by IGF-I, and their role in transducing the mitogenic stimulus to the cell nucleus, have been partly unraveled. Binding of IGF-I to its receptor leads to autophosphorylation of the receptor and activation of its tyrosine kinase activity. An important substrate for the receptor is insulin-receptor substrate-1 (IRS-1) (Izumi *et al.* 1987, Shemer *et al.* 1987) which, after phosphorylation, serves as a docking site for multiple src homology-2 (SH2)-domain containing proteins, such as p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3-K) (Myers *et al.* 1992,

Backer *et al.* 1993), and the guanine-nucleotide exchange factor Grb2/Sos (Baltensperger *et al.* 1993, Skolnik *et al.* 1993 *a,b*). Among the downstream targets of PI3-K are protein kinase B (PKB) (Burgering & Coffey 1995), p70^{S6K} (Cheatham *et al.* 1994), and the small GTPases Rho, Rac and cdc42 (Chou & Blenis 1996). On the other hand, interaction of the receptor with Grb2/Sos results in activation of the ras-raf-MAP (mitogen-activated protein) kinase pathway (Cobb & Goldsmith 1995). In previous work (Dufourny *et al.* 1997), we showed that in MCF-7 cells the IGF-I induced rise in cyclin D1 steady-state levels is inhibited by the specific PI3-K inhibitors LY294002 (Vlahos *et al.* 1994) and wortmannin (Arcaro & Wymann 1993). The MAP kinase/ERK kinase 1 (MEK1) inhibitor PD098059 (Dudley *et al.* 1995) does not affect cyclin D1 mRNA and protein expression induced by IGF-I. This suggests that in MCF-7 cells proliferative IGF-I signaling is dependent on PI3-K activity, and that the MAP kinases are not involved in this process. Here we investigate the mechanism by which PI3-K regulates the expression of cyclin D1. Theoretically, the observed increase in cyclin D1 levels upon IGF-I stimulation may be caused by transcriptional activation, by increased mRNA stability, by enhanced translation efficiency and by increased protein half-life. Contradictory reports on this issue have appeared in the literature. In NIH3T3 fibroblasts, it was found that rapamycin, an inhibitor of p70^{S6K} (Price *et al.* 1992), reduces the cyclin D1 mRNA half-life (Hashemolhosseini *et al.* 1998). This would suggest a role for a PI3-K-dependent signaling route in the regulation of cyclin D1 mRNA stability. In contrast, it has been reported that inhibition of PI3-K activity reduces the efficiency of translation of cyclin D1 mRNA in MCF-7 and MCF-10A cells (Muise-Helmericks *et al.* 1998). Here we show that activation of the PI3-K pathway stabilizes the 4.5 kb cyclin D1 mRNA, whereas the stability of the 1.5 kb mRNA is not influenced. The observed stabilization of the 4.5 kb mRNA species is not a general phenomenon, since other rapidly regulated and unstable mRNAs, such as those encoding *c-fos*, *c-jun* and *c-myc* are not stabilized upon activation of the PI3-K pathway.

Materials and Methods

Reagents

LY294002 was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). Cycloheximide was from Sigma (St Louis, MO, USA) and actinomycin D was supplied by Boehringer Mannheim GmbH (Mannheim, Germany). The anti-cyclin D1 antibody H-295 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cyclin D1 promoter-luciferase construct was kindly provided by Dr Rolf Müller (Marburg, Germany).

Hybridization probes

The cyclin D1 probe, a kind gift from Dr D Beach (Cold Spring Harbor, NY, USA), is a 1325 bp PCR product encoding the entire human cyclin D1 cDNA sequence (Xiong *et al.* 1991). The *c-fos* probe is a 1800 bp EcoRI–XhoI human *c-fos* cDNA fragment. The *c-jun* probe is a 1350 bp EcoRI–BamHI human *c-jun* cDNA fragment. The *c-myc* probe is a 800 bp HindIII–ClaI human *c-myc* cDNA fragment. The 28S rRNA oligonucleotide has the following sequence: 5′-AAC GAT CAG AGT AGT GGT ATT TCA CC-3′.

Cell culture

MCF-7 human breast cancer cells were cultured in Dulbecco's Minimal Essential Medium/Ham's F12 medium (1:1), supplemented with 5% fetal calf serum, 300 µg/ml glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were made quiescent by culturing them for 24 h in phenol red-free medium containing 5% dextran-coated charcoal-treated serum, followed by a 24 h incubation in phenol red-free, serum-free medium, supplemented with 0.2% BSA, 10 µg/ml transferrin and 30 nM sodium selenite.

PKB kinase assay

Cells were washed in PBS and lysed in IP buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 0.1 mM Na₃VO₄). Lysates were pre-cleared by incubation with 25 µl 10% protein A-agarose beads pre-coupled to normal rabbit serum for 15 min at 4 °C. Lysates were then incubated with 70 µl 10% protein A-agarose beads, pre-coupled to anti-PKB antibody for 2 h at 4 °C. Beads were washed twice in IP buffer, twice in 50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 0.5 M LiCl, 200 µM Na₃VO₄, and once in 50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 200 µM Na₃VO₄. Beads were then resuspended in a 25 µl kinase mix (50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 50 µM ATP) supplemented with 5 µg histone 2B and 5 µCi [γ -³²P]ATP per sample. The mixture was incubated for 20 min at 30 °C, after which the reaction was stopped with 8 µl of 4 × sample buffer (200 mM Tris–HCl pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol). The suspension was boiled and centrifuged, and the supernatant was run on a 15% SDS-polyacrylamide gel. The gel was dried and exposed to X-ray film (Fuji, Tokyo, Japan).

Western blotting

Experiments were performed in six-well plates or in 25 cm² flasks. Cells were washed in ice-cold PBS and lysed in 100 µl lysis buffer per well or 200 µl per flask

(25 mM Tris-phosphate pH 7.8, 15% glycerol, 1% Triton X-100). Lysates were cleared by centrifugation and protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein (5 µg) were size-separated in a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose. The membrane was stained with Ponceau S (Sigma), to check for equal loading, and blocked in 1% BSA for 1 h. Incubation with the anti-cyclin D1 antibody was performed overnight at 4 °C (1:2500 in 0.1% BSA in TBST (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.2% Tween 20)). The membrane was washed in TBST and exposed to horseradish peroxidase-coupled anti-rabbit antibody for 1 h (1:8000 in TBST). For detection, the Enhanced Chemiluminescence (ECL) system (NEN Life Science Products Inc., Boston, MA, USA) was used.

Northern blotting

Experiments were performed in 25 cm² flasks. Cells were washed in ice-cold PBS and lysed in RNazol B (Biotecx Laboratories Inc., Houston, TX, USA). Total RNA was extracted and precipitated according to the manufacturer's instructions and dissolved in sterile water. Equal amounts of RNA (10 µg) were glyoxylated in 50% Me₂SO and size-separated by electrophoresis in a 0.8% agarose gel. RNA was transferred to a nylon membrane (Genescreen, NEN Life Science Products) and cross-linked to the membrane by u.v. irradiation. The blots were pre-hybridized for several hours at 42 °C in 50% deionized formamide, 1 M NaCl, 50 mM Tris-HCl pH 7.5, 0.2% BSA, 0.2% polyvinylpyrrolidone, 0.2% Ficoll 400, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate and 200 µg/ml denatured herring sperm DNA. Hybridization was performed overnight in the same solution to which the specific ³²P-labeled cDNA probes (10⁶ c.p.m./ml) were added. Double-stranded probes were labeled by random priming using Rediprime (Amersham Life Sciences Inc., Little Chalfont, UK). The single-stranded 28S rRNA oligonucleotide was end-labeled using [α -³²P]dATP and T4 polynucleotide kinase. Membranes were exposed to X-ray film (Fuji).

Transfections

Cells were seeded in six-well plates (10⁶ cells/plate) one day before transfection. Transfections were done using the DAC30 transfection reagent (Eurogentec, Seraing, Belgium). Per well, 3 µg DAC30, 0.5 µg cyclin D1 promoter-luciferase construct and 0.1 µg cytomegalovirus (CMV)-*lacZ*-encoding plasmid was used. For each well, DNA was diluted in 500 µl HBS buffer (150 mM NaCl, 20 mM Hepes, pH 7.4). DAC30 was diluted in an equal volume of HBS. The DNA- and DAC30 dilutions were then combined, gently mixed and left at room temperature for 20 min. Immediately before transfection, the medium

on the cells was refreshed using 1 ml per well. The DNA/DAC30 mixture was now added dropwise to the cells under continuous swirling, after which they were incubated for 3 h at 37 °C. After transfection, the transfection mixture was replaced by phenol red-free, serum-free medium, with or without LY294002, for 24 h.

Luciferase assay

Approximately 24 h after transfection, the culture medium was removed and the cells were washed in ice-cold PBS and lysed in 100 µl lysis buffer (25 mM Tris-phosphate pH 7.8, 15% glycerol, 1% Triton X-100, 1 mM DTT and 8 mM MgCl₂) per well. Lysates were centrifuged for 2 min at 14 000 r.p.m. and 25 µl supernatant were used in the assay. Luciferase activities were measured using a Lumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany), and were corrected for transfection efficiency using β -galactosidase activity from the cotransfected *lacZ* plasmid as an internal standard.

LacZ assay

Of the lysate, 10 µl were mixed with 221 µl 100 mM sodium phosphate buffer (pH 7.4), 66 µl *o*-nitrophenyl- β -D-galactopyranoside (ONPG, 4 mg/ml) and 3 µl 4.5 M β -mercaptoethanol/0.1 M MgCl₂. After incubation for 30–60 min at 37 °C, reactions were stopped with 500 µl 0.5 M Na₂CO₃. Reaction mixtures were extracted with 500 µl chloroform/isoamylalcohol (19:1). After 2 min of centrifugation at 14 000 r.p.m., the absorbance at 420 nm of the upper phase was measured spectrophotometrically.

Results

Transcriptional activation of cyclin D1 by IGF-I, but not by TPA and E2, is mediated by PI3-K

We have previously shown that addition of 10 ng/ml IGF-I to the medium of serum-starved MCF-7 cells increases cyclin D1 mRNA and protein levels. This increase is inhibited by the PI3-K inhibitor LY294002, indicating that PI3-K activity is needed for the observed rise in cyclin D1 level. We have extended this observation by showing activation of the cyclin D1 promoter by constitutively active PI3-K mutants as well as by Rac and cdc42, two related small GTPases functioning downstream of PI3-K (submitted for publication). We then investigated whether LY294002-mediated inhibition is also observed with other agents known to increase cyclin D1 levels in these cells. As demonstrated in Fig. 1A, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA, 100 ng/ml), the steroid hormone E2 (1 nM) and epidermal growth factor (EGF, 40 ng/ml), like IGF-I, increase cyclin D1 protein levels when added to serum-deprived MCF-7 cells. TPA, a strong growth inhibitor for

Figure 1 Effect of PI3-K inhibition on cyclin D1 protein induction. A. Serum-starved MCF-7 cells were stimulated with 40 ng/ml IGF-I, 100 ng/ml TPA, 1 nM E2 or 40 ng/ml EGF with or without pre-incubation with 50 μM LY294002 for 10 min. Cells were harvested 5 h after stimulation and cyclin D1 protein levels were analyzed by Western blotting. B. Activation of PKB by several stimuli. Serum-starved cells were stimulated with 2 or 10 ng/ml IGF-I, 100 ng/ml TPA or 1 nM E2 for 7 min. Cells were lysed, PKB was immunoprecipitated and its kinase activity was determined using histone 2B as a substrate.

MCF-7 cells because of its induction of large amounts of p21^{waf1} (Dufourny *et al.* 1997), has been shown to enhance cyclin D1 promoter activity via an activating protein (AP)-1 response element at position -954 relative to the transcription start site (Albanese *et al.* 1995, Watanabe *et al.* 1996). Activation of AP-1 element-binding transcription factors, e.g. *c-fos* and *c-jun*, is thought to result from activation of protein kinase C, and subsequent induction of the raf-MEK1-MAP kinase signaling cascade by TPA. The same is true for EGF, which is also a strong activator of the MAP kinases. E2 has been shown to activate the cyclin D1 promoter through an Estrogen Response Element (ERE) located more than 940 bp upstream of the transcription start site (Altucci *et al.* 1996). Surprisingly, the increase in cyclin D1 protein levels caused by TPA or E2 as well as that by EGF is inhibited, as in the case of IGF-I, by the prior addition of LY294002 (50 μM) to the medium of serum-starved MCF-7 cells (Fig. 1A). The signaling pathways activated by TPA and E2 have been extensively studied in recent years. These studies indicate that activation of PI3-K by these agents is not a generally accepted concept. To determine whether TPA and/or E2 is/are able to enhance PI3-K activity in MCF-7 cells, we measured the activity of PKB/Akt-1, a well-documented downstream target of PI3-K (Burgering & Coffey 1995). Serum-starved MCF-7 cells were incubated with 2 or 10 ng/ml IGF-I, 1 nM E2 or 100 ng/ml TPA. After 7 min at 37 °C, cells were lysed. PKB was immunoprecipitated and its kinase activity was measured using histone 2B as a substrate. Whereas IGF-I in relatively low concentrations already activates PKB, no activity above the level found in untreated, serum-starved

Figure 2 Effect of PI3-K inhibition on cyclin D1 mRNA steady-state levels in quiescent cells. Serum-starved MCF-7 cells were treated with 50 μM LY294002. Cells were harvested 4 h after the addition and cyclin D1 mRNA levels were analyzed by Northern blotting. Equal loading was checked for by hybridization of the Northern blot to a glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) probe.

cells was detected after treatment with TPA or E2 (Fig. 1B). Conceivably, an essential basal transcription factor might be controlled by PI3-K in such a way that cyclin D1 transcription is turned off when PI3-K is inhibited by LY294002. However, in transient transfection assays, employing a -1170 to +141 cyclin D1 promoter-luciferase construct, no effect of LY294002 on the basal activity of the promoter was observed (data not shown). The clear effect of LY294002 on cyclin D1 levels observed after treatment with IGF-I, TPA and E2, combined with the lack of PI3-K activation by TPA and E2, thus strongly suggests the existence of an efficient PI3-K-dependent mechanism controlling cyclin D1 levels post-transcriptionally.

PI3-K activity is required for cyclin D1 mRNA stabilization

Theoretically, the observed enhancing effect of PI3-K activity on cyclin D1 levels, which is independent of the presence of transcriptional activators, may be explained by stabilization of the transcript, enhancement of the rate of translation, or stabilization of the cyclin D1 protein itself. As illustrated in Fig. 2, we reproducibly observed a considerable drop in the steady-state level of the 4.5 kb cyclin D1 mRNA in serum-starved cells during a 4 h period after addition of LY294002, whereas the amount of the 1.5 kb mRNA species remained unchanged. This observation suggests that inhibition of PI3-K results in destabilization of cyclin D1 mRNA and that the long mRNA species is more sensitive to the destabilizing effect.

Figure 3 Effect of PI3-K inhibition on decay of cyclin D1 mRNA and protein. Serum-starved cells were stimulated for 4 h with 40 ng/ml IGF-I. After stimulation, cells were given fresh serum-free medium (A+B) or serum-free medium containing 20 μg/ml actinomycin D (C+D) with or without 50 μM LY294002. Cells were harvested at the indicated time points (in h) and cyclin D1 mRNA and protein levels were determined by Northern (A+C) and Western (B+D) blotting. All RNA samples are shown in duplicate, and equal loading was checked for by hybridization of the Northern blots to a 28S rRNA specific oligonucleotide.

To test this hypothesis, we studied the decay of cyclin D1 mRNA in MCF-7 cells in the presence and absence of the PI3-K inhibitor. Quiescent cells were stimulated with IGF-I for 4 h. After incubation, the cells were given fresh serum-free medium with or without 50 μM LY294002. Cells were harvested at the indicated time points and cyclin D1 mRNA and protein levels were analyzed (Fig. 3A and B). In the absence of LY294002, the levels of the 4.5 kb cyclin D1 mRNA were quite stable. Only a slight decrease in its steady-state level was observed during the course of the experiment. In contrast, in the presence of LY294002 the levels of the 4.5 kb mRNA dropped much more rapidly and were already below the basal steady-state level after 3 h. The 1.5 kb mRNA level was more constant throughout the experiment, also in the presence of LY294002. The level of the short mRNA diminished only slightly after 3 h but this was insignificant when compared with the decrease in the level of the long mRNA. The rate of decay of the 4.5 kb mRNA corresponded quite well with what was seen in the corresponding Western blot analysis. The cyclin D1 protein level was

rather constant over a 4.5 h period in the untreated cells, whereas a rapid decline was observed in cells treated with LY294002 (Fig. 3B).

Cyclin D1 mRNA stabilization by PI3-K does not require de novo transcription

To determine whether this PI3-K mediated stabilizing influence on the 4.5 kb cyclin D1 mRNA requires *de novo* transcription, we repeated the above experiment but included actinomycin D to inhibit transcription. After stimulation with IGF-I, the cells were given fresh serum-free medium containing 20 μg/ml actinomycin D with or without 50 μM LY294002. Cells were harvested after 2 and 4 h, and cyclin D1 mRNA and protein levels were determined. The results are shown in Fig. 3C and D. In agreement with the data in Fig. 3A and B, again in this experiment no difference in mRNA and protein levels in absence or presence of LY294002 was detected after 2 h. However, after 4 h the decrease in the 4.5 kb cyclin D1 mRNA level, as well as in the protein level, was

Figure 4 Effect of PI3-K inhibition on half-life of several early gene-encoding mRNAs. A. Serum-starved MCF-7 cells were stimulated for 45 min with 10 ng/ml TPA. After stimulation, cells were given fresh serum-free medium with or without 25 μ M LY294002. Cells were harvested at the indicated time points (in min) and *c-fos* and *c-jun* mRNA levels were determined by Northern blotting. B. Quiescent cells were stimulated for 4 h with 20 ng/ml IGF-I, after which the cells were given fresh serum-free medium with or without 50 μ M LY294002. Cells were harvested at the indicated time points (in h) and *c-myc* mRNA levels were analyzed by Northern blotting. Equal loading was checked for by hybridization of the Northern blots to a 28S rRNA-specific oligonucleotide.

significantly stronger in the presence of LY294002 than in its absence. Again, the effect was much less pronounced if not absent for the 1.5 kb cyclin D1 mRNA species. These data indicate that the stabilizing effect of PI3-K activity on the long cyclin D1 mRNA does not require *de novo* transcription, since it is also observed in the presence of actinomycin D.

Stabilization of mRNA by PI3-K is specific for cyclin D1

The 4.5 kb cyclin D1 mRNA species possesses about 3 kb 3'-untranslated region (3'-UTR), containing a cluster of six AUUUA repeats within a region of 420 residues (Motokura *et al.* 1991). The 1.5 kb cyclin D1 mRNA only has about 200 residues in its 3'-UTR and lacks the AUUUA repeats. The AUUUA sequences are known as instability determinants (Ross 1995), and are present in many rapidly regulated transcripts (Henics *et al.* 1994, Mayo *et al.* 1995, Kastelic *et al.* 1996, Peng *et al.* 1996, Chagnovich & Cohn 1997, Wang *et al.* 1997, Esnault *et al.* 1998). To determine whether other transcripts, containing AUUUA repeats in their 3'-UTR, are influenced by PI3-K in the same way, we studied the effect of LY294002 on *c-fos*, *c-jun* and *c-myc* mRNA stability in MCF-7 cells. These early response gene-encoding transcripts all possess 3'-UTRs containing AUUUA repeats. The levels of *c-fos* and *c-jun* mRNA were

induced by stimulation with 10 ng/ml TPA for 45 min. After stimulation, cells were given fresh serum-free medium with or without 25 μ M LY294002 and harvested at the indicated time points. The results are shown in Fig. 4A. TPA treatment dramatically increases the mRNA levels of both *c-fos* and *c-jun*. In the absence of LY294002, both mRNAs decay quite rapidly. Within 1 h their levels decrease to that of unstimulated cells. However, when LY294002 is present these mRNAs have a significantly longer half-life. After 2.5 h the levels of *c-fos* and *c-jun* mRNA are still clearly above the basal level in untreated, serum-starved cells. These data suggest that inhibition of PI3-K stabilizes these messengers and that PI3-K activity has a destabilizing influence. In Fig. 4B, the results are shown for *c-myc* mRNA. In this experiment, cells were stimulated for 4 h with IGF-I. After stimulation, cells were given fresh, serum-free medium with or without LY294002 and cells were harvested 3 and 5 h later. At both time points, no difference in *c-myc* mRNA levels was observed in the presence or absence of LY294002, suggesting that PI3-K activity does not influence *c-myc* mRNA stability. Although the mechanisms of these PI3-K-mediated effects are still completely unknown, these data indicate that the stabilizing influence of PI3-K on cyclin D1 mRNA is not a general phenomenon for all unstable mRNAs.

Figure 5 Decay of cyclin D1 protein after inhibition of transcription or translation. Quiescent MCF-7 cells were stimulated for 4 h with 20 ng/ml IGF-I. After stimulation, 20 μ g/ml actinomycin D (A) or 50 μ g/ml cycloheximide (B) was added with or without 50 μ M LY294002 (LY). Cells were harvested at the indicated time points (in h for A, in min for B) and cyclin D1 protein levels were analyzed by Western blotting.

PI3-K activity does not affect cyclin D1 protein stability

In several experiments we observed that the decrease in steady state-levels in the presence of LY294002 was more pronounced for the cyclin D1 protein level than it was for the cyclin D1 mRNA level (see Fig. 3). Since we could not exclude from the previous experiments the possibility that PI3-K activity influences cyclin D1 protein stability in addition to mRNA stability, we also studied cyclin D1 protein levels in the presence of the translation inhibitor cycloheximide. Serum-starved MCF-7 cells were stimulated with 20 ng/ml IGF-I for 4 h. After stimulation, 20 μ g/ml actinomycin D or 50 μ g/ml cycloheximide was added in combination with 50 μ M LY294002. Subsequently, cells were harvested at 2 h intervals and cyclin D1 protein levels were monitored by Western blotting. Figure 5A shows that, in agreement with the data in Fig. 3B, addition of actinomycin D alone does not affect cyclin D1 protein levels, no decrease being observed during the course of the experiment. In contrast, when LY294002 is present cyclin D1 protein levels drop significantly. After 4 h, the level is already below that in quiescent cells. In contrast, in the presence of cycloheximide cyclin D1 protein levels drop to almost undetectable levels within 2 h, even in the absence of LY294002, indicating that the protein is short-lived (data not shown). Thus an effect of LY294002, if any, on cyclin D1 protein stability could not be determined. Therefore, we repeated the above experiment but instead harvested the cells at much earlier time points. As can be seen in Fig. 5B, 45 min after the addition of cycloheximide cyclin D1 protein levels dropped almost to basal level, and after

60 min basal level was reached. There were no differences in the decay rates in the presence or absence of LY294002. From this we conclude that the cyclin D1 protein is rather unstable, its half-life being less than 45 min irrespective of the presence or absence of LY294002. Thus, the observed decrease in steady-state levels after 4–6 h in the presence of LY294002 is not likely to have been caused by reduced stability of the cyclin D1 protein.

Discussion

Upregulation of cyclin D1 expression is the first cell cycle-related event after growth-factor stimulation of quiescent cells. In MCF-7 breast cancer cells, moderate overexpression of cyclin D1 has been shown to be sufficient for cell growth under low-serum conditions, establishing cyclin D1 as the key mediator of G1/S-phase transition (Zwijsen *et al.* 1996). However, the signal-transduction pathways regulating cyclin D1 expression are still poorly understood. Here we demonstrate that the PI3-K signaling pathway, besides being able to activate the cyclin D1 promoter, is involved in post-transcriptional regulation of cyclin D1, stabilizing the 4.5 kb cyclin D1 mRNA. Inhibition of PI3-K activity by LY294002 clearly reduces the half-life of this mRNA species, whereas the minor 1.5 kb cyclin D1 mRNA is much less affected. This stabilizing influence of PI3-K is not a general phenomenon for all unstable mRNAs, since inhibition of PI3-K has no effect at all on *c-myc* mRNA stability and has an opposite (stabilizing) effect on *c-fos* and *c-jun* mRNAs. PI3-K does not seem to play a role in regulating cyclin D1 protein stability in our MCF-7 cell model system.

In several experiments we observed that the destabilizing effect of LY294002 was more pronounced for the cyclin D1 protein level than it was for the mRNA level. This could not be explained by an additional effect of LY294002 on protein stability besides mRNA stability, since we have clearly shown that LY294002 does not influence cyclin D1 protein stability. Furthermore, LY294002 predominantly affects the 4.5 kb transcript and hardly influences the stability of the 1.5 kb mRNA species. When both mRNAs are translated with an equal efficiency, giving rise to the same protein, the destabilizing effect would be expected to be less clear for the protein level than for the mRNA level. A possible explanation is that the 1.5 kb messenger is not, or is very inefficiently, translated, so that almost all cyclin D1 protein is derived from the 4.5 kb mRNA. It may even be that not all of the 4.5 kb cyclin D1 mRNA is translated, and that the protein is derived from a restricted pool of this longer mRNA species. Whether or not a difference in translation efficiency between the two cyclin D1 mRNAs exists, and how this is brought about, requires further investigation.

PI3-K-dependent signaling routes have been implicated in the regulation of cyclin D1 expression levels in a limited

number of earlier reports. In NIH3T3 fibroblasts, a PI3-K-dependent signaling route has also been reported to be involved in regulation of cyclin D1 mRNA stability (Hashemolhosseini *et al.* 1998). This pathway encompasses p70^{S6K}, a known downstream target of PI3-K. In this study two cyclin D1 mRNAs were described, one of them being the 4.5 kb species, also found in MCF-7 cells, the other one being about 4 kb in length. The shorter form of 1.5 kb was not mentioned in this study. The half-lives of both messengers were diminished by treatment of the cells with rapamycin, a specific inhibitor of p70^{S6K}. In addition, an effect of this PI3-K/p70^{S6K}-dependent pathway on cyclin D1 protein stability was also claimed. However, this effect is much less clear, and the authors found an unusually long half-life (about 2 h) of the cyclin D1 protein in these experiments. Besides regulation of cyclin D1 mRNA and protein stability, PI3-K-mediated regulation of translation of cyclin D1 mRNA has also been described. Muise-Helmericks *et al.* (1998) have described an effect of a PI3-K-dependent signaling route on the expression level of D-type cyclins in MCF-7 cells. This concerns the effect of herbimycin A, one of the ansamycin, which causes a reduction in the synthetic rate of D-type cyclins prior to changes in mRNA expression. Since (in our experiments) LY294002 clearly influences the steady-state level of cyclin D1 mRNA, this effect is probably unrelated to our findings. A stabilizing influence of activation of PI3-K on a specific mRNA species has been reported before. Ionomycin-induced stabilization of interleukin (IL)-3 mRNA in mast cells is counteracted by the PI3-K inhibitor wortmannin. c-Jun N-terminal kinase (JNK) was shown to be the downstream target of PI3-K required for this stabilization. The *cis*-acting elements were shown to be so-called AU-rich regions (AREs) in the 3'-UTR (Ming *et al.* 1998). These AREs contain clusters of AUUUA pentamer repeats, which are well-known instability determinants for mRNAs. They are often present in the 3'-UTRs of short-lived, rapidly regulated mRNAs, such as the mRNAs for the cytokines IL-1 (Kastelic *et al.* 1996), IL-2 (Henics *et al.* 1994) and IL-3 (Mayo *et al.* 1995), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Esnault *et al.* 1998), tumor necrosis factor- α (TNF- α) (Wang *et al.* 1997), and for the early genes *c-fos* (Chagnovich & Cohn 1997), *c-jun* (Peng *et al.* 1996) and *c-myc* (Chagnovich & Cohn 1997). The AREs have been shown to be responsible for the short half-lives of these transcripts. Several studies have shown that mutant mRNAs, for example GM-CSF (Esnault *et al.* 1998) or epidermal growth-factor receptor (McCulloch *et al.* 1998), which lack their natural AREs have increased half-lives. Moreover, linking of the 3'-UTRs of such unstable mRNAs to coding sequences of, for example, the luciferase or β -globin mRNAs, severely reduces the half-lives of these normally very stable messengers (Wang *et al.* 1997). The 4.5 kb cyclin D1 mRNA species contains about 3 kb of 3'-UTR which is absent from the 1.5 kb

transcript. The 5'-UTR and coding region are identical in both mRNAs. The 3'-UTR contains six AUUUA repeats clustered in a region of 420 bp (Motokura *et al.* 1991). By analogy with what has been found for the IL-3 mRNA, the AU-rich regions may constitute the *cis*-acting elements required for PI3-K-mediated stabilization of the longer cyclin D1 transcript. Current investigations aim to determine whether or not this is the case.

Some evidence indicates that (over)expression of shorter variants of cyclin D1 mRNA, lacking (part of) the 3'-UTR, is correlated with transformation and malignancy. Several studies have reported on the existence of mutant cyclin D1 mRNA species in malignant cells of leukemia patients (Withers *et al.* 1991, Seto *et al.* 1992, Hosokawa *et al.* 1998). These mutations are either deletions or truncations caused by genomic deletion or chromosomal translocation. In all cases the AREs are no longer present in the cyclin D1 transcripts. However, in these studies no experiments were performed to determine the effects of these mutations on cyclin D1 mRNA stability. The MDA MB-453 breast cancer cell line has been reported to possess a normal and a truncated copy of the cyclin D1 gene. The truncated copy is amplified threefold, resulting in overexpression of a short cyclin D1 mRNA which corresponds to the 1.5 kb species in MCF-7 cells. This mRNA has a longer half-life than its 4.5 kb counterpart, indicating that sequences in the 3'-UTR influence cyclin D1 mRNA stability (Lebwohl *et al.* 1994). PI3-K-mediated stabilization of cyclin D1 mRNA may account for the observed decrease in cyclin D1 steady-state levels when LY294002 is added to the cells, irrespective of the presence and nature of transcriptional activators.

As summarized in the model in Fig. 6, the hormones IGF-I, EGF and E2 as well as the phorbol ester TPA are all capable of activating the cyclin D1 promoter, each via their own responsive element. E2, in complex with its receptor, binds to the ERE (Altucci *et al.* 1996), whereas TPA and EGF activate the p42/44 MAP kinases, which results in increased expression of the early response genes *c-fos* and *c-jun*. The *c-fos* and *c-jun* proteins, in turn, form heterodimeric complexes and bind to the AP-1 binding site (Albanese *et al.* 1995, Watanabe *et al.* 1996). IGF-I activates PI3-K, which, as we recently showed, is able to induce cyclin D1 promoter activity via a pathway involving the small GTPases Rac and cdc42 on the one hand (submitted for publication), and stabilizes the 4.5 kb cyclin D1 mRNA on the other. Although transcriptional activation of the cyclin D1 promoter by E2, EGF and TPA does not involve PI3-K activity, the steady-state levels of cyclin D1 mRNA do not become elevated when LY294002 is present, since basal PI3-K activity is required for stabilization of the 4.5 kb mRNA. This model may explain the fact that the ras-raf-MAP kinase signaling route is considered to be the essential pathway for inducing proliferation in many cell types. In these studies, often

Figure 6 Schematic representation of a model explaining LY-mediated inhibition of cyclin D1 expression induced by different stimulators. Inhibition of PI3-K activity by LY294002 not only blocks signaling to the cyclin D1 promoter (e.g. by IGF-I) but also destabilizes the predominant 4.5 kb cyclin D1 mRNA. This leads to lower cyclin D1 steady-state levels. See the text for further discussion.

the effects only of specific MEK1/MAP kinase inhibitors have been determined. When the MAP kinases are important in transcriptional activation of the cyclin D1 promoter, as is the case for TPA and EGF in our MCF-7 cell system, these MAP kinase inhibitors will undoubtedly inhibit the proliferative response. Since, however, in most of these studies specific PI3-K inhibitors have not been used, the possibility that a certain basal level of PI3-K activity is necessary for proliferation as well cannot be excluded. Furthermore, some inhibitors of the ras-raf-MAP kinase route, e.g. the farnesyltransferase inhibitor and the ansamycins, geldamycin and herbimycin A, are, according to some recent reports (Muisse-Helmericks *et al.* 1998, Law *et al.* 1999), also capable of inhibiting the PI3-K pathway. Therefore, inhibition of proliferation by these agents can no longer be considered as evidence for the exclusive importance of the MAP kinase pathway for proliferation.

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