THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 12, Issue of March 22, pp. 9684–9689, 2002
Printed in U.S.A.

Glycogen Synthase Kinase-3 Couples AKT-dependent Signaling to the Regulation of p21^{Cip1} Degradation*

Received for publication, July 2, 2001, and in revised form, November 8, 2001
Published, JBC Papers in Press, January 4, 2002, DOI 10.1074/jbc.M106157200

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Signaling via the phosphoinositide 3-kinase (PI3K)/AKT pathway is crucial for the regulation of endothelial cell (EC) proliferation and survival, which involves the AKT-dependent phosphorylation of the DNA repair protein p21^{Cip1} at Thr-145. Because p21^{Cip1} is a short-lived protein with a high proteasomal degradation rate, we investigated the regulation of p21^{Cip1} protein levels by PI3K/AKT-dependent signaling. The PI3K inhibitors Ly294002 and wortmannin reduced p21^{Cip1} protein abundance in human umbilical vein EC. However, mutation of the AKT site Thr-145 into aspartate (T145D) did not increase its protein half-life. We therefore investigated whether a kinase downstream of AKT regulates p21^{Cip1} protein levels. In various cell types, AKT phosphorylates and inhibits glycogen synthase kinase-3 (GSK-3). Upon serum stimulation of EC, GSK-3β was phosphorylated at Ser-9. Site-directed mutagenesis revealed that GSK-3 *in vitro* phosphorylated p21^{Cip1} specifically at Thr-57 within the Cdk binding domain. Overexpression of GSK-3β decreased p21^{Cip1} protein levels in EC, whereas the specific inhibition of GSK-3 with lithium chloride interfered with p21^{Cip1} degradation and increased p21^{Cip1} protein about 10-fold in EC and cardiac myocytes (30 mM, $p < 0.001$). These data indicate that GSK-3 triggers p21^{Cip1} degradation. In contrast, stimulation of AKT increases p21^{Cip1} via inhibitory phosphorylation of GSK-3.

p21^{Cip1} regulates cell cycle progression and confers apoptosis protection in endothelial cells. Initially considered as an inhibitor of proliferation, increasing evidence now suggests that p21^{Cip1} plays a complex role for cell differentiation and survival in development as well as in senescence (1). Likewise, an increasingly complex picture of the regulation of p21^{Cip1} has emerged. In addition to the transcriptional induction by p53-dependent (2) and -independent mechanisms (reviewed in Ref. 3), ubiquitin-mediated (4) as well as ubiquitin-independent proteasomal degradation processes (5, 6) regulate p21^{Cip1} protein stability. Various proteins engage in protein-protein interactions with p21^{Cip1} (1) and may thereby influence p21^{Cip1} degradation. Thus, complex formation processes involving the C terminus of p21^{Cip1}, which directly binds to the 20 S proteasome (6), have been implicated in the regulation of p21^{Cip1} turnover, particularly via interaction with the DNA adapter protein, PCNA[1] (7). Because p21^{Cip1} is a phosphoprotein targeted for post-translational modification by various kinases, phosphorylation of p21^{Cip1} may regulate the interaction of p21^{Cip1} with its binding partners and, thus, could also affect p21^{Cip1} stability. Importantly, phosphorylation of p21^{Cip1} at Ser-146 and at Thr-145 was shown to modulate the p21^{Cip1}-PCNA complex formation *in vitro* (8) and in human endothelial cells, respectively (9).

Protein kinase B/AKT regulates growth, survival, and metabolism in response to phosphatidylinositol-3 kinase (PI3K) activation of a variety of cells. Among the downstream messengers that mediate AKT-derived signals in hypertrophy (10), glycogen synthesis (11), survival (12), and proliferation (13), glycogen synthase kinase-3 (GSK-3) α/β is phosphorylated by AKT at Ser-21/Ser-9, which inactivates GSK-3 kinase activity (11). AKT also phosphorylates p21^{Cip1} in HER-2/*neu*-overexpressing (14) as well as in native human endothelial cells (9), in which phosphorylation by AKT regulates p21^{Cip1} binding to PCNA (9). Functionally, signaling via PI3K/AKT is involved in the regulation p21^{Cip1}, because PI3K inhibitors prevent p21^{Cip1} accumulation in response to DNA damage in fibroblasts and leukemia cells (15) and overexpression of AKT induces p21^{Cip1} in muscle cells (16, 17). However, it is unknown how AKT controls p21^{Cip1} protein levels. Therefore, we investigated the signaling pathways underlying the regulation of p21^{Cip1} protein turnover by PI3K/AKT-dependent signaling. We have identified p21^{Cip1} as a substrate for GSK-3β-mediated phosphorylation, the inhibition of which stabilizes p21^{Cip1} protein. This interaction provides a novel post-translational regulatory element coupling AKT to p21^{Cip1}.

MATERIALS AND METHODS

Endothelial Cell Culture, Materials, and Immunoblotting—Human umbilical vein endothelial cells (HUVEC) were purchased from Cell Systems/Clonetics (Solingen, Germany) and were cultured as described previously (18). For experiments, cells were used at the third passage. Ly294002 was from Biomol (Hamburg, Germany), lactacystin was bought from Calbiochem, and wortmannin and cycloheximide were from Sigma. Following cell lysis, HUVEC homogenates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were then incubated with antibodies against c-*myc* (Santa Cruz Biotechnology), actin (Sigma), phospho-GSK-3α/β (Ser-21/Ser-9) (Cell Signaling, Beverly, MA), or p21 (BD Transduction Laboratories).

Plasmids and Transfection—The plasmid encoding human p21^{Cip1} was cloned by PCR into the pcDNA3.1-*Myc*-His vector (InVitrogen). To

* This study was supported by grants from the Deutsche Forschungsgemeinschaft (Di600 2-3/4 and SFB 553 C2). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a Young Investigator's grant from the University of Frankfurt.

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[1] The abbreviations used are: PCNA, proliferating cell nuclear antigen; PI3K, phosphoinositide 3-kinase; GSK-3, glycogen synthase kinase-3; HUVEC, human umbilical vein endothelial cells; BrdUrd, bromodeoxyuridine; EC, endothelial cell(s); CHX, cycloheximide; LiCl, lithium chloride; Cdk, cyclin-dependent kinase.

engineer various p21^{Cip1} constructs defective in potential acceptor sites for GSK-3-dependent phosphorylation, threonines and serines were changed by site-directed mutagenesis (Stratagene) to alanines as indicated. The plasmid encoding bovine AKT was kindly donated by Dr. J. Downward and subcloned into the pcDNA3.1 vector as described (18). To engineer a constitutively active AKT construct (AKT S/T), Ser-473 and Thr-308 were changed to aspartic acid by site-directed mutagenesis. GSK-3β was cloned into the pcDNA3.1 vector. Clones with verified sequences were used to transfect HUVEC (3.5×10^5cells/6 cm well, 3 μg of plasmid DNA, 25 μl of Superfect) or COS-7 cells (3.8×10^5 cells/6 cm plate, 8 μg of plasmid DNA, 30 μl Superfect) as described previously (18).

In Vitro Kinase Assay—For detection of GSK-3 phosphorylation of p21^{Cip1} *in vitro*, COS-7 cells were transfected with *myc*-tagged p21^{Cip1} wild type or p21^{Cip1} constructs, and overexpressed p21^{Cip1}-*myc* was immunoprecipitated from whole cell lysates (500 μg/sample) using antibodies against the *myc* tag. Then, p21^{Cip1} immunoprecipitates were incubated with recombinant GSK-3β (New England Biolabs) for 30 min at 30 °C in 30 μl of a kinase reaction mixture containing 20 mM Tris (pH 7.5), 5 mM dithiothreitol, 10 mM $MgCl_2$, 5 μM ATP, and 5 μCi of [γ-^{32}P]ATP. The reaction was terminated by the addition of SDS-sample dye, and samples were subjected to 12% SDS-PAGE and analyzed by autoradiography. Similarly, *myc*-tagged and overexpressed p27^{Kip1} was immunoprecipitated and assayed for GSK-3-dependent phosphorylation.

Cell Cycle Analysis—For quantification of cell proliferation rates, cells were incubated with 10 μM bromodeoxyuridine (BrdUrd) for 1 h, and incorporated BrdUrd was detected by enzyme-linked immunosorbent assay according to the instructions of the manufacturer (Roche Molecular Biochemicals). To distinguish between individual cell cycle phases, HUVEC were incubated with BrdUrd and subsequently stained with anti-BrdUrd fluorescent antibodies (BD PharMingen) as well as with the DNA dye 7-amino-actinomycin D according to the manufacturer's instructions. Then, flow cytometry of these cells was performed using a FACSCalibur cell sorter (Becton Dickinson).

Statistics—Data are expressed as the mean ± S.E. from at least three independent experiments. Two treatment groups were compared by the independent samples *t* test and three or more groups by one-way analysis-of-variance followed by *post hoc* analysis adjusted with an LSD correction for multiple comparisons (SPSS).

RESULTS

Regulation of p21^{Cip1} Protein Levels by PI3K—p21^{Cip1} stabilization upon DNA damage depends on the activity of the PI3K (15). Likewise, in human EC, the PI3K inhibitor Ly294002 time-dependently decreased p21^{Cip1} protein levels (Fig. 1*A*). Similar results were obtained with another inhibitor of PI3K, wortmannin (data not shown). Coincubation with the proteasome inhibitor lactacystin completely prevented the PI3K inhibitor-induced decrease in p21^{Cip1} protein (Fig. 1*A*), suggesting that the down-regulation of p21^{Cip1} following PI3K inhibition involved proteasomal degradation processes. Because we and others have recently demonstrated that AKT phosphorylates p21^{Cip1} in native EC (9) as well as in an HER-2/*neu*-overexpressing cell line (14), we investigated whether the phosphorylation of p21^{Cip1} by AKT regulates p21^{Cip1} protein half-life following inhibition of protein synthesis with cycloheximide (CHX). Under serum-containing conditions, the protein half-life of the AKT-phosphomimetic p21^{Cip1} construct, in which Thr-145 was changed to Asp (T145D), was not increased compared with p21^{Cip1} wild type (Fig. 1, *B* and *C*). These data suggest that direct phosphorylation by AKT at Thr-145 does not account for the PI3K-dependent p21^{Cip1} protein stabilization. Therefore, we hypothesized that signaling events downstream of AKT might mediate the observed effect of PI3K inhibition on p21^{Cip1} protein levels.

Regulation of p21^{Cip1} by GSK-3—In various cell types, AKT phosphorylates and inhibits the kinase activity of GSK-3 (11), which regulates the proteasomal degradation of cyclin D (13). To explore whether GSK-3 could also be involved in the regulation of p21^{Cip1} protein expression, we investigated whether GSK-3 phosphorylates p21^{Cip1}. Recombinant GSK-3β induced p21^{Cip1} phosphorylation *in vitro* (Fig. 2*A*). Mutagenesis of the

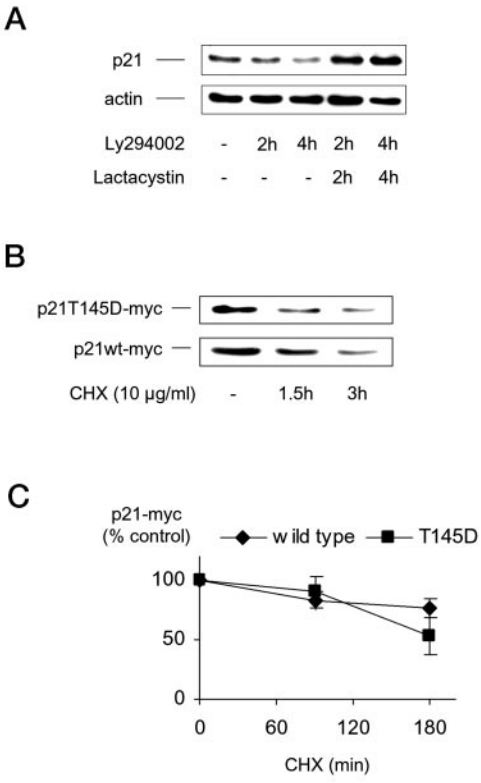

FIG. 1. **Regulation of p21^{Cip1} protein levels by PI3K independent of p21^{Cip1} phosphorylation at Thr-145.** *A*, time-dependent effect of the PI3K inhibitor Ly294002 (10 μM) on endogenous p21^{Cip1} levels in HUVEC in the absence and presence of the proteasome inhibitor lactacystin (5 μM). *Lower panel*, actin reprobe to indicate equal loading of the gel. *B*, protein half-life of the AKT-phosphomimetic p21^{Cip1} construct (T145D, *upper panel*) *versus* p21^{Cip1} wild type (*wt*) assessed by inhibition of protein synthesis with CHX (10 μg/ml). *C*, densitometric analysis of protein half-life of the p21^{Cip1} constructs. Mean ± S.E. of n = 3–5 individual experiments; ■, p21^{Cip1} T145D; ♦, p21^{Cip1} wild type.

AKT-phospho-acceptor site, Thr-145, had a minor effect on GSK-3-mediated phosphorylation (Fig. 2*A*). Likewise, p21^{Cip1} phosphorylation by GSK-3 was unchanged by replacement of the reported phospho-acceptor site for PKC-dependent phosphorylation, Ser-146 (8) (Fig. 2*A*), indicating that an amino acid different from the already described phosphorylation sites of p21^{Cip1} serves as acceptor site for GSK-3-mediated phosphorylation. To identify the specific site for GSK-3-mediated phosphorylation, we further analyzed the GSK-3 kinase activity toward two constructs of p21^{Cip1} in which the two potential sites for GSK-3-dependent phosphorylation within the p21^{Cip1} amino acid sequence matching the reported GSK-3 consensus sequence, (S/T)*XXX***P**(S/T), *i.e.* Ser-114 and Ser-27, were mutated to alanine (underlined characters indicate the actual acceptor amino acids for phosphorylation). As shown in Fig. 2*B*, GSK-3 equally phosphorylated these constructs when compared with p21^{Cip1} wild type, excluding Ser-114 and Ser-27 as the p21^{Cip1} acceptor site for GSK-3-dependent phosphorylation. Therefore, we took a systematic approach to find the specific GSK-3 phosphorylation site and engineered a C-terminally truncated p21^{Cip1} construct consisting of amino acids 1–109 only, which was still phosphorylated by recombinant GSK-3 (data not shown), indicating an N-terminal site for GSK-3 phosphorylation. We then systematically changed the remaining N-terminal 10 serines and threonines to alanine and found that only the mutation of Thr-57 to Ala resulted in a complete lack of phosphorylation by GSK-3 (Fig. 2, *B* and *C*). We conclude, therefore, that GSK-3 specifically phosphorylates p21^{Cip1} at Thr-57. Because the N-terminal sequence of p21^{Cip1} shares a

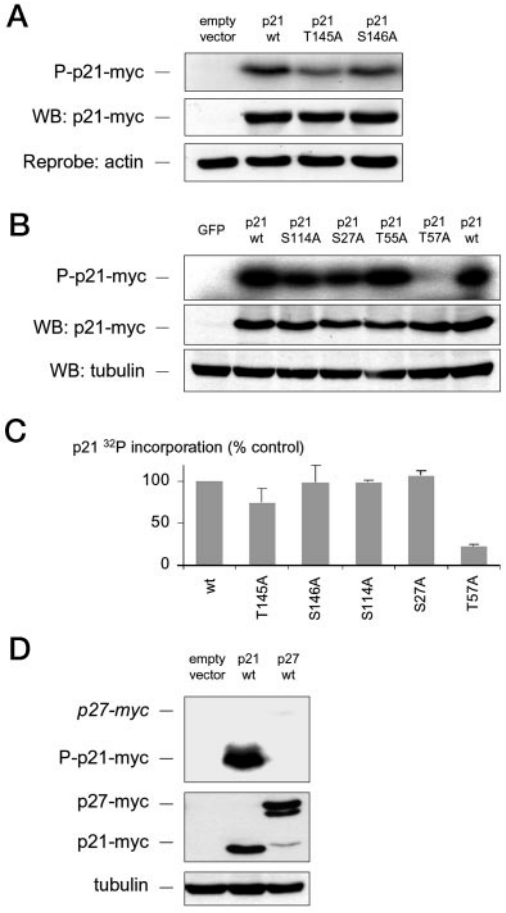

FIG. 2. **Phosphorylation of p21^{Cip1} by GSK-3 *in vitro*.** Anti-c-*myc* immunoprecipitates from COS-7 cells transfected with empty vector or green fluorescent protein (*GFP, left lanes*) or with *myc*-tagged wild type p21^{Cip1} or the indicated constructs were incubated with human recombinant GSK-3β and [γ-^{32}P]ATP, separated by SDS-PAGE, and incorporated γ^{32}P was visualized by autoradiography (*upper panels*). *Middle panels*, Western blot analysis to indicate the expression levels of p21^{Cip1} constructs; *lower panels*, actin or tubulin immunostainings of the gels to demonstrate equal loading. *A*, GSK-3-mediated phosphorylation of wild type p21^{Cip1} (*p21 wt*) *versus* T145A and S146A constructs. *B*, GSK-3-mediated phosphorylation of p21 wild type *versus* S114A, S27A, T55A, and T57A constructs. *C*, densitometric analysis of p21^{Cip1} phosphorylation by GSK-3 from $n = 3$ individual experiments. *D*, GSK-3-mediated phosphorylation of wild type p21^{Cip1} *versus* wild type p27^{Kip1}.

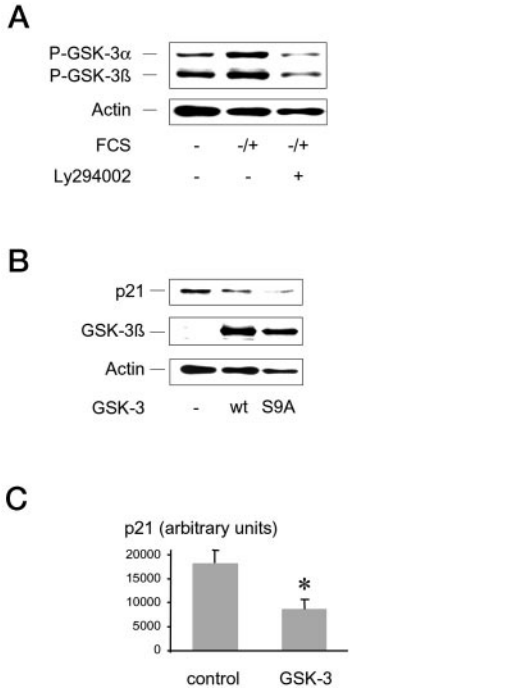

FIG. 3. **Regulation of p21^{Cip1} by GSK-3 in intact cells.** *A*, effect of serum readmission for 1 h following serum starvation (9 h) in the absence or presence of Ly294002 (10 μM, 10 min preincubation) on GSK-3 phosphorylation in HUVEC. *Upper panel*, Western blot analysis with a phospho-specific antibody against GSK-3α and -β isoforms phosphorylated at Ser-21 and Ser-9, respectively; *lower panel*, actin reprobe of the gel to demonstrate equal loading. *B*, Western blot analysis of endogenous p21^{Cip1} protein in HUVEC overexpressing wild type GSK-3β or the AKT-phospho-resistant construct GSK-3β S9A (*versus* empty vector, *upper panel*). *Middle panel*, c-*myc* reprobe to indicate GSK-3β-*myc* expression; *lower panel*, actin reprobe to confirm equal loading of the gel. *C*, densitometric analysis of p21^{Cip1} protein levels in HUVEC overexpressing GSK-3β wild type *versus* empty vector (*left bar*). *, $p < 0.02$.

great homology with p27^{Kip1}, we tested whether GSK-3 also phosphorylates a *myc*-tagged p27^{Kip1} construct. However, as shown in Fig. 2*C*, GSK-3 selectively phosphorylates p21^{Cip1} but not p27^{Kip1}.

Next, we investigated whether GSK-3 is subject to phosphorylation in EC exposed to stimuli that are known to activate AKT. Cells were starved in a growth factor-depleted medium, which time-dependently reduced GSK-3α and-β phosphorylation at Ser-21 and Ser-9, respectively (data not shown). Serum readmission induced a marked increase in both phosphorylated GSK-3α and β isoforms (Fig. 3*A*), which was completely suppressed in the presence of the PI3K inhibitor Ly294002 (Fig. 3*A*). Thus, serum stimulation of EC induces the PI3K-dependent phosphorylation of GSK-3. We then explored whether GSK-3 is involved in the regulation of p21^{Cip1} protein expression in EC. Overexpression of GSK-3β wild type decreased p21^{Cip1} protein expression (Fig. 3, *B* and *C*). Likewise, transfection of the GSK-3β S9A construct, which cannot be phosphorylated at the phosphoacceptor site for AKT-dependent phosphorylation, Ser-9, and thus cannot be inhibited by AKT-coupled signaling, significantly suppressed endogenous p21^{Cip1} protein levels in EC (Fig. 3*B*). These data suggest that GSK-3 regulates p21^{Cip1} protein expression downstream of PI3K/AKT in EC.

Regulation of p21^{Cip1} Protein Expression by the GSK-3 Inhibitor LiCl—To investigate whether phosphorylation by GSK-3 mediates the GSK-3-dependent regulation of p21^{Cip1}, we determined the effect of the specific GSK-3 inhibitor, lithium chloride (LiCl) (19, 20), on p21^{Cip1} protein expression. LiCl (1–30 mM) led to a dose- and time-dependent increase in endogenous p21^{Cip1} protein levels in EC (Fig. 4, *A–D*). This finding indicates that interference with GSK-3 activity in EC increases p21^{Cip1} protein expression. A similar increase in p21^{Cip1} protein levels occurred following LiCl treatment of freshly isolated rat cardiomyocytes, whereas p21^{Cip1} protein remained unchanged following LiCl treatment of SV40-transformed COS-7 cells (Fig. 4*E*). Because p21^{Cip1} is a target for direct phosphorylation by GSK-3, we determined whether the observed increase in p21^{Cip1} resulted from a post-translational effect on p21^{Cip1} protein stability when protein synthesis was blocked by the addition of CHX. LiCl treatment significantly increased protein stability rate of p21^{Cip1} in EC (Fig. 5, *A* and *B*). Likewise, LiCl also stabilized p21^{Cip1} degradation in the presence of the transcription inhibitor actinomycin D (Fig. 5*C*), suggesting that the observed effect of LiCl on p21^{Cip1} protein levels is mainly due to the post-transcriptional stabilization of p21^{Cip1}. Importantly, LiCl treatment also increased the stability of the AKT-phosphomimetic p21^{Cip1} T145D construct (Fig. 5*D*). To determine the contribution of GSK-3-dependent Thr-57 phosphorylation to the regulation of p21^{Cip1} degradation, we overexpressed the p21^{Cip1} T57A construct in HUVEC. As

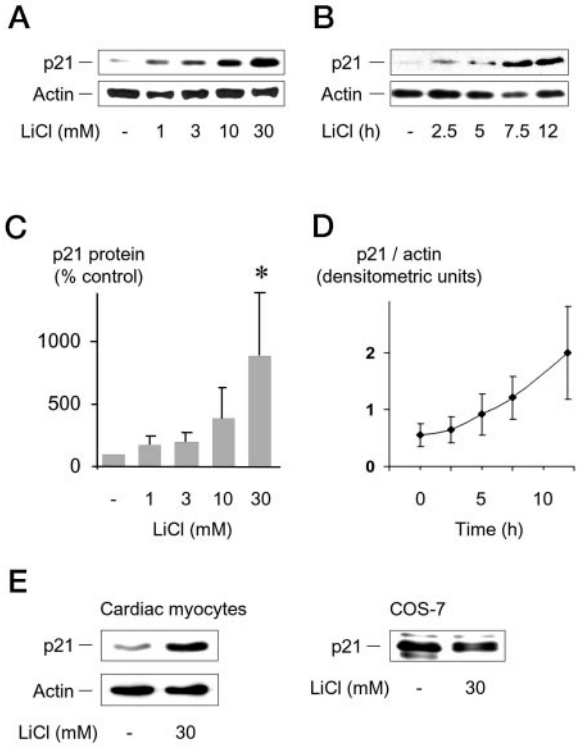

FIG. 4. **Time- and dose-dependent up-regulation of p21^{Cip1} by lithium chloride.** *A* and *C*, HUVEC were incubated with LiCl for 18 h at the indicated doses and lysed, and endogenous p21^{Cip1} protein levels were determined. *A*, representative Western blot. *C*, densitometric analysis of $n = 3$–5 independent experiments; *, $p < 0.001$. *B* and *D*, HUVEC were incubated with 30 mM LiCl for the indicated times before assessment of endogenous p21^{Cip1} protein. *B*, representative Western blot. *D*, densitometric analysis of $n = 4$ independent experiments. *E*, Western blot analysis of p21^{Cip1} protein levels following incubation of the indicated cell type with LiCl (30 mM, 18 h). *Left panel*, cardiac myocytes; *right panel*, COS-7, a transformed kidney cell line.

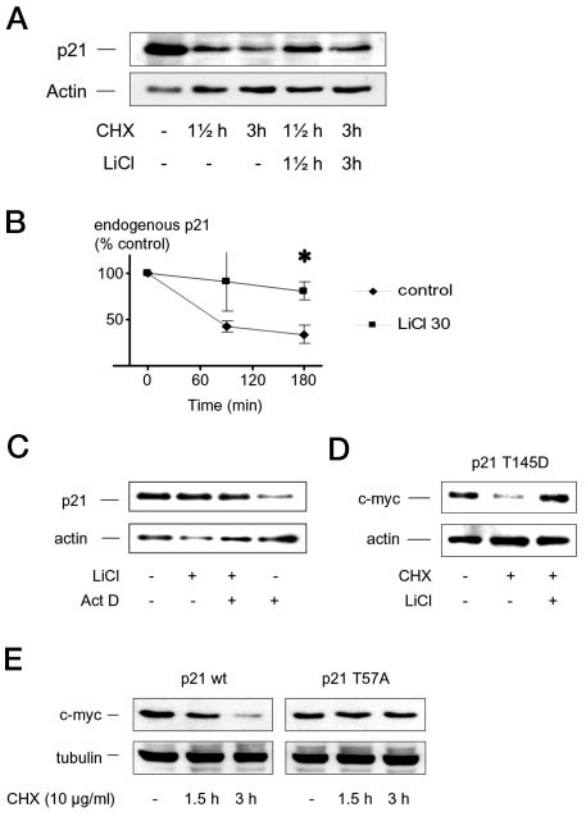

FIG. 5. **Stabilization of p21^{Cip1} protein by lithium chloride.** Assessment of p21^{Cip1} half-life following incubation of HUVEC with CHX (10 µg/ml) for 90 and 180 min in the absence and presence of LiCl (30 mM). *A*, representative Western blot and *B*, densitometric analysis of $n = 4$ individual experiments; ♦, control; ■, 30 mM LiCl; *, $p < 0.001$. *C*, p21^{Cip1} protein levels determined by Western blot analysis following incubation with the inhibitor of transcription, actinomycin D (*Act D*, 7.5 µg/ml, 150 min, *lane 4*). LiCl (30 mM) was added 10 min following act D (*lane 3*). *D*, Western blot analysis of overexpressed AKT-phosphomimetic (*T145D*) p21^{Cip1} protein stability following inhibition of protein synthesis with CHX (10 µg/ml, 180 min) in the presence or absence of LiCl (30 mM, added shortly after CHX). *E*, Western blot analysis of protein degradation of p21 wild type (*wt*, *upper left*) *versus* T57A mutant (*upper right*) following CHX (10 µg/ml) for the indicated times. *Lower panels*, tubulin expression to indicate equal loading of the gel.

shown in Fig. 5*E*, interference with Thr-57 phosphorylation significantly reduced p21^{Cip1} degradation following 1.5 and 3 h of cycloheximide treatment ($p < 0.01$). Thus, GSK-3-mediated phosphorylation regulates p21^{Cip1} post-transcriptionally at the level of protein stabilization.

Effect of LiCl on EC Proliferation—Finally, we analyzed the functional effect of GSK-3-mediated p21^{Cip1} protein stabilization in EC. The increase in p21^{Cip1} protein following LiCl treatment at concentrations of 3 or 10 mM LiCl was not associated with a marked decrease in proliferation rates as assessed by BrdUrd incorporation as well as by flow cytometry (Fig. 6, *A* and *B*). However, 30 mM LiCl arrested EC specifically in the G_2/M phase of the cell cycle (Fig. 6, *A* and *B*). Overexpression of constitutively active AKT completely reversed the cell cycle inhibitory activity of LiCl even at a concentration of 30 mM (Fig. 6*C*), confirming previous data on the modulation of the cell cycle regulatory properties of p21^{Cip1} by AKT-dependent phosphorylation (9). Morphological analysis by phase contrast microscopy revealed a hypertrophic appearance of the cells following an 18-h incubation with LiCl (30 mM, not shown).

DISCUSSION

Herein we have investigated the regulation of p21^{Cip1} by PI3K/AKT-dependent signaling in EC. Pharmacological inhibition of PI3K reduces p21^{Cip1} protein levels, confirming previously published data from human leukemia and mouse primary embryo fibroblasts (15, 21). However, direct phosphorylation of p21^{Cip1} by AKT, which is known to abrogate p21^{Cip1} binding to PCNA, did not significantly affect protein degradation. In contrast, phosphorylation by GSK-3, which undergoes PI3K-dependent inhibitory phosphorylation in response to serum stimulation, regulates protein turnover of p21^{Cip1} because 1) pharmacological inhibition of GSK-3 dramatically increases p21^{Cip1} protein half-life, and 2) phospho-negative mutagenesis of the GSK-3 phosphorylation site Thr-57 interferes with p21^{Cip1} degradation.

The GSK-3 phosphorylation site, Thr-57, does not match the reported consensus motif for GSK-3-dependent phosphorylation characterized by a priming phosphorylation at amino acid +4 upstream of the GSK-3-phosphorylated acceptor site. This finding is consistent with previous reports describing GSK-3-dependent phosphorylation of target substrates at regions different from the (S/T)*XXX***P**(S/T) motif (13, 22). Roach (see Ref. 23 for review) has proposed previously that any phosphorylated amino acid may in dependence on the tertiary protein structure sterically relate to a potential phospho-acceptor site to enhance its affinity toward phosphorylation by GSK-3. Others have reported that GSK-3 may, in addition to this so-called "priming substrate concept," also act as a proline-directed kinase. Therefore, although our data do not exclude an initial phosphorylation anywhere at the molecule by another kinase, proline at position 58 of the p21^{Cip1} amino acid sequence neighboring Thr-57 suggests that GSK-3 could indeed act on p21^{Cip1} as a

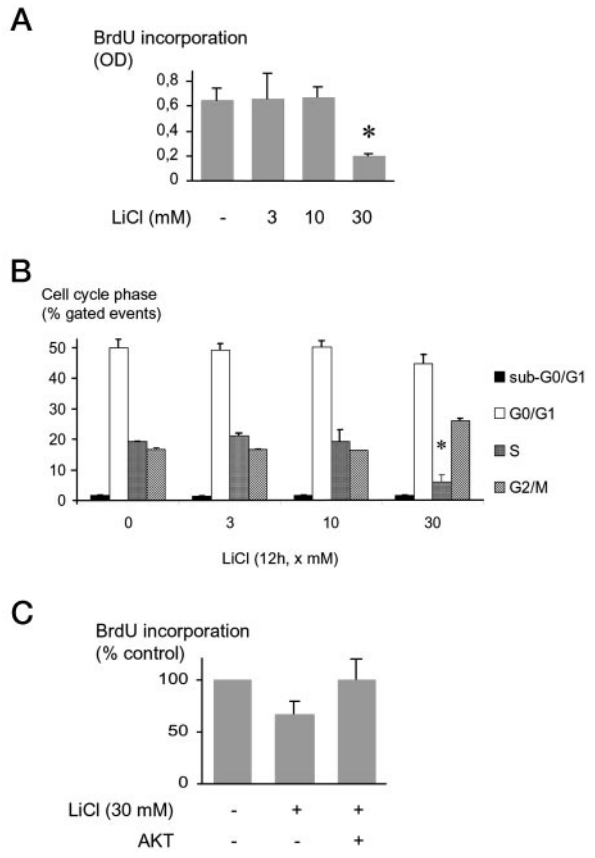

FIG. 6. **Regulation of EC proliferation by lithium chloride.** *A*, dose-dependent effect of LiCl (18 h) on EC proliferation assessed by BrdUrd incorporation; *, $p < 0.05$. *B*, dose-dependent effect of LiCl (12 h) on EC proliferation assessed by flow cytometry; *, $p < 0.05$. *C*, effect of LiCl (30 mM, 15 h) on BrdUrd incorporation in HUVEC overexpressing the constitutively active AKT construct T308D/S473D (*right bar*) *versus* empty vector (*middle bar*).

proline-directed kinase rather than via "processive" phosphorylation. Because GSK-3 is phylogenetically closely related to the cyclin-dependent kinases (Cdks) and the herein reported GSK-3 phosphorylation site, Thr-57, is located within the Cdk-binding region of p21^{Cip1}, one might also speculate that a potential interaction between p21^{Cip1} and GSK-3 could facilitate GSK-3 phosphorylation of p21^{Cip1}. Such a mechanism has previously been reported to underlie the phosphorylation of axin and β-catenin by GSK-3 (24). Currently, experiments are being carried out to characterize the molecular interaction between p21^{Cip1} and GSK-3 more in detail. Interestingly, the GSK-3 phosphorylation site, Thr-57, is highly conserved among various mammalian species. However, although the N-terminal domains of p21^{Cip1} and p27^{Kip1} share close homology, Thr-57 is not present in p27^{Kip1}, suggesting that the post-translational interaction of p21^{Cip1} with GSK-3 could contribute to the functional diversity of both cell cycle regulatory proteins.

The GSK-3 phosphorylation site, Thr-57, is located in the Cdk binding region of p21^{Cip1}. Cayrol and Ducommun (7) have previously reported that a p21^{Cip1} construct defective in Cdk binding is characterized functionally by an increased half-life because of a reduced sensitivity toward proteasome-mediated hydrolysis. Indeed, co-immunoprecipitation experiments demonstrate decreased Cdk4 binding by the p21^{Cip1} T57A construct,[2] suggesting a potential mechanism that could underlie the stabilization of p21^{Cip1} toward proteasomal degradation following GSK-3 inhibition. In addition, protein-protein interactions involving the C terminus of p21^{Cip1} were previously suggested to regulate its proteasomal turnover rate (6). In particular, a p21^{Cip1} construct defective in PCNA binding (M147A) was shown to undergo accelerated turnover compared with native p21^{Cip1} (6, 7). This is consistent with our finding that AKT-phosphomimetic mutation (p21^{Cip1} T145D), which results in PCNA release from complex formation with p21^{Cip1} (9), did not increase p21^{Cip1} half-life compared with overexpressed wild type. Only recently, the capability of p21^{Cip1} to bind with its C terminus to the C8 interaction domain of the 20 S proteasome has been shown to be of major relevance for the regulation of p21^{Cip1} protein degradation, proving that mechanisms beyond PCNA binding are dominant over the stabilizing influence of PCNA on p21^{Cip1} half-life (6). Although we show that GSK-3 directly phosphorylates p21^{Cip1} at its N-terminal domain, this post-translational modification could also sterically influence the C terminus and thus trigger p21^{Cip1} degradation via modulation of the interaction of p21^{Cip1} with the proteasome. Thereby, a concept of phosphorylation-dependent regulation of p21^{Cip1} turnover resembles the mechanism by which GSK-3-mediated phosphorylation triggers both nuclear exit and subsequently the ubiquitin-mediated proteasomal degradation of another regulator of G_1 cell cycle progression, cyclin D (13, 25). While preparing this manuscript, Mao *et al.* (26) published that LiCl stabilized p53 and thereby transcriptionally increased p21^{Cip1} mRNA and protein in endothelial cells. In contrast, another study (27) reported that p53 mRNA expression is down-regulated following long-term treatment with LiCl. Our data clearly demonstrate that LiCl up-regulates p21^{Cip1} protein expression in the presence of the transcription inhibitor actinomycin D as well as when protein translation was inhibited by CHX. Moreover, LiCl also increased the levels of transfected p21^{Cip1}, suggesting that LiCl can regulate p21^{Cip1} independently of p53. Further investigations are warranted to explore the detailed molecular mechanisms coupling GSK-3-dependent phosphorylation at Thr-57 to the regulation of p21^{Cip1} protein stability.

Recent findings have linked AKT signaling to the regulation of p21^{Cip1}. 1) PI3K post-transcriptionally regulates p21^{Cip1} accumulation in response to DNA damage (15). 2) p21^{Cip1} is required for the induction of smooth muscle cell proliferation in response to the AKT-coupled platelet-derived growth factor (28). 3) Overexpression of AKT induces p21^{Cip1} in muscle cells (16, 17). 4) AKT directly phosphorylates p21^{Cip1} in HER-2/*neu*-overexpressing (14) as well as in native human endothelial cells (9), and 5) AKT phosphorylation regulates p21^{Cip1} binding to PCNA (9). Here, we show that AKT-coupled activation regulates p21^{Cip1} protein half-life as a summary effect. Via phosphorylation and thus inhibition of GSK-3, AKT indirectly interferes with the degradation-triggering p21^{Cip1} phosphorylation by GSK-3, which may compensate for and even prevail over the destabilizing effect of PCNA release (7) associated with the direct phosphorylation of p21^{Cip1} by AKT *in vivo* (9). But what is the physiological effect of p21^{Cip1} protein up-regulation in response to AKT? We have recently provided evidence that direct phosphorylation of p21^{Cip1} at Thr-145 by AKT modulates the cell cycle regulatory properties of p21^{Cip1} as Thr-145-phosphomimetic p21^{Cip1} does not any longer bind to PCNA, less readily engages in the inhibitory complex formation with Cdk2, and importantly, promotes the activating assembly of Cdk4 with cyclin D that leads to exit of the cell cycle out of G_0 (9). Thus, AKT activation modifies p21^{Cip1} function to cooperate with cyclin D, which AKT also up-regulates, to allow for G_1 progression. Taking into account the function of p21^{Cip1} in DNA repair and genome stability, by ensuring suffic-

[2] L. Rössig, unpublished results.

ient amounts of p21^{Cip1}, cell cycle progression under AKT control might represent a safe-guarded mode of proliferation compared with p21^{Cip1}-deficient proliferating cells, which are prone to apoptosis (29). Preliminary data indeed suggest that AKT-dependent phosphorylation of p21^{Cip1} also regulates the accessibility of Dnmt1 for DNA.[2]

In summary, our data show that under resting conditions, endogenous GSK-3β kinase activity reduces p21^{Cip1} levels by phosphorylation-dependent degradation of p21^{Cip1}. Inhibition of GSK-3 upon PI3K/AKT activation abrogates GSK3-mediated p21^{Cip1} phosphorylation at Thr-57 and increases p21^{Cip1} half-life, providing a novel post-translational interaction coupling AKT to p21^{Cip1}.

Acknowledgments—We are grateful to Rebeca Salguero Palacios, Christiane Mildner-Rihm, and Susanne Ficus for expert technical assistance.

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