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Phosphorylation by Cyclin C/Cyclin-Dependent Kinase 2 following Mitogenic Stimulation of Murine Fibroblasts Inhibits Transcriptional Activity of LSF during G_1 Progression▽

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Transcription factor LSF is required for progression from quiescence through the cell cycle, regulating thymidylate synthase (*Tyms*) expression at the G_1/S boundary. Given the constant level of LSF protein from G_0 through S, we investigated whether LSF is regulated by phosphorylation in G_1. In vitro, LSF is phosphorylated by cyclin E/cyclin-dependent kinase 2 (CDK2), cyclin C/CDK2, and cyclin C/CDK3, predominantly on S309. Phosphorylation of LSF on S309 is maximal 1 to 2 h after mitogenic stimulation of quiescent mouse fibroblasts. This phosphorylation is mediated by cyclin C-dependent kinases, as shown by coimmunoprecipitation of LSF and cyclin C in early G_1 and by abrogation of LSF S309 phosphorylation upon suppression of cyclin C with short interfering RNA. Although mouse fibroblasts lack functional CDK3 (the partner of cyclin C in early G_1 in human cells), CDK2 compensates for this absence. By transient transfection assays, phosphorylation at S309, mediated by cyclin C overexpression, inhibits LSF transactivation. Moreover, overexpression of cyclin C and CDK3 inhibits induction of endogenous *Tyms* expression at the G_1/S transition. These results identify LSF as only the second known target (in addition to pRb) of cyclin C/CDK activity during progression from quiescence to early G_1. Unexpectedly, this phosphorylation prevents induction of LSF target genes until late G_1.

The transcription factor LSF (late simian virus 40 factor [20], also named CP2 [24], LBP-1c [53], and SEF [6]) is ubiquitously expressed in all cell types (32, 44). LSF is essential for cell cycle progression at the G_1/S transition after reentry of quiescent cells into the cell cycle, substantially through its regulation of thymidylate synthase (*Tyms*) gene expression (31). Disruption of LSF function can lead either to apoptosis in S phase (31) or to cell cycle arrest at the G_1/S transition (11), both phenotypes being relieved by a source of exogenous thymidine, thereby circumventing the requirement for thymidylate synthase activity.

In investigating how LSF activity could be modulated during cell cycle progression, we previously demonstrated that LSF is targeted by the MEK/extracellular signal-regulated kinase (ERK) signaling pathway, which is critical for reentry of quiescent cells into the G_1 phase of the cell cycle (35). ERK phosphorylates LSF at S291 upon mitogenic stimulation of multiple cell types, including mouse fibroblasts and primary peripheral human T lymphocytes (30, 49). Signaling pathways essential for the subsequent progression through G_1 into S phase include the G_1 cyclin-dependent kinases (CDKs) (38). These include the extensively investigated D-type cyclins and their associated kinases CDK4/6 as well as E-type cyclins associated with CDK2 (39, 41). Recently, an additional cyclin/CDK complex, cyclin C/CDK3, was also shown to facilitate efficient exit of human cells from G_0 into the cell cycle (33, 36). Cyclin C was not previously thought to be a regulator of cell cycle progression but was instead demonstrated to complex with CDK8 to phosphorylate the C-terminal domain of the largest subunit of RNA polymerase II (23). However, cyclin C-associated kinase activity peaks shortly after mitogenic stimulation of a quiescent human cell line, where it forms complexes with CDK3. One critical target of this growth-stimulated kinase is pRb (33).

Given that LSF induces expression of *Tyms* at the G_1/S transition (31), we hypothesized that LSF activity might be regulated during G_1 phase by a G_1 cyclin/CDK complex. We originally anticipated that LSF activity would be upregulated by phosphorylation in late G_1. Here, we present evidence that LSF is, indeed, an in vivo target of a G_1 cyclin/CDK in growth-regulated mouse fibroblasts. Unexpectedly, however, it is transiently phosphorylated at S309 by cyclin C/CDK2 in early G_1 following growth factor stimulation of quiescent cells. As determined by transient transfection assays using reporter constructs, this phosphorylation at S309 (as well as phosphorylation at S291) inhibits, rather than activating, LSF transactivation potential. LSF is then dephosphorylated at both sites as cells progress through G_1, prior to the G_1/S boundary. If such dephosphorylation is prevented, induction of *Tyms* is inhibited. It thus appears that phosphorylation by both ERK and cyclin C/CDK2 at the G_0/G_1 transition downmodulates the activity of LSF during passage through G_1, until induction of *Tyms* at the G_1/S boundary.

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MATERIALS AND METHODS

Cell culture, synchronization, and analysis of cellular DNA content. NIH 3T3 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (JRH Biosciences), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were growth arrested by incubation in DMEM containing 0.5% calf serum for 36 h and stimulated to reenter the cell cycle by addition of medium containing either 10% calf serum or 10 ng/ml epidermal growth factor (EGF). When indicated, cells were treated with either 10 μM MEK inhibitor U0126 (Cell Signaling Technology) or dimethyl sulfoxide (the vehicle) for 30 min prior to stimulation. Both 293T cells and primary *Cdk2*$^{+/-}$ and *Cdk2*$^{-/-}$ murine embryonic fibroblasts (MEFs) (5) were propagated in DMEM supplemented with 10% fetal bovine serum (FBS) (HyClone), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. MEFs were growth arrested in DMEM containing 0.1% FBS for 72 h and stimulated to reenter the cell cycle by medium containing 10% FBS. The retroviral packaging cell line Phoenix-Eco (ATCC) was propagated in DMEM supplemented with 10% heat-inactivated calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

To analyze cellular DNA content, trypsinized cells were fixed with ethanol, treated with RNase A, and stained with propidium iodide (Sigma). DNA content was analyzed using FlowJo software (Tree Star, Inc.) and a Becton Dickinson FACSCalibur flow cytometer, with gating on single cells by use of an FL2 area-versus-width dot plot.

Plasmid constructs. pQE30-LSF (42) and pEF1α-LSF (14, 31) have been described previously. The parental plasmid pCMV-QZ was generated by inserting a linker to introduce a BamHI site into HindIII- and XhoI- digested pcDNA1/Amp (Invitrogen). pCMV-LSF was constructed by inserting LSF cDNA into the BamHI and XhoI sites of pCMV-QZ (Q. Zhu and U. Hansen, unpublished). Substitution mutant constructs of pCMV-LSF were generated via PCR-mediated site-directed mutagenesis, using a QuikChange kit (Stratagene). The mutations included S289A (TCC to GCC), S291A (TCA to GCA), S309A (TCA to GCA), S291E (TCA to GAA), S309D (TCA to GAC), and T258A (ACA to GCA). The mutant LSF cDNA regions were subcloned by fragment exchange into the pQE30-LSF backbone. pCMV-LSF FLAG was constructed by replacing the LSF stop codon in pCMV-LSF with a HindIII site by PCR and inserting the amplified product into pCMV-Tag4A (Stratagene) digested with BamHI and HindIII.

Flag-tagged human cyclin C in pCMV-Bam-Neo and hemagglutinin (HA)-tagged human CDK3 in pRcCMV (33), pCMV-neo-Bam-p21 (4), the CDK2 expression vector pCMV-Neo-Bam CDK2 (46), and the bacterial expression plasmid pGST-pRb (379-928) (13) have been described previously. The expression plasmid pcDNA3-p27 was a gift from Anindya Dutta. The C-terminal Flag-tagged cyclin E expression vector (pCMV-cyclin E-Flag) was constructed by replacing the cyclin E stop codon in pRcCMV-cyclin E (18) with a XhoI site, introducing a BamHI site at the 5′ end by PCR, and inserting the amplified, digested product into pCMV-Tag4A.

The reporter plasmid pGL3B-WT4E1b was constructed by digesting pWT4E1b (53) with BamHI and XhoI and cloning the fragment containing the E1b TATA sequences and four tandem LSF binding sites from the human immunodeficiency virus type 1 long terminal repeat into pGL3-Basic (Promega) digested with BglII and XhoI.

The retroviral vector LZRSpBMN-linker-IRES-EGFP, a derivative of the pBMN-I-GFP vector containing the EBNA-1 gene and the puromycin resistance cassette from pLZRS-LacZ(A) (21), was a gift from Gary Nolan. LSF cDNA from pCMV-LSF-S309A was inserted into the BamHI and XhoI sites of LZRSpBMN-linker-IRES-EGFP to construct LZRSpBMN-linker-IRES-EGFP-LSF-S309A. The retroviral vectors LZRSpBMN-linker-IRES-EGFP-Cyclin C-FLAG and LZRSpBMN-linker-IRES-EGFP-Cdk3-HA were constructed by amplifying the cDNAs from pCMV-Bam-Neo-Cyclin C-FLAG and pRcCMV-CDK3-HA, respectively, and inserting them into the BamHI and XhoI sites of pLZRS-IRES-EGFP. The envelope vector pCL-Eco has been described previously (12).

Extract preparation. Whole-cell extracts were prepared by lysing cells in TD buffer (50 mM HEPES [pH 7.5], 250 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, Complete Mini EDTA-free protease inhibitor cocktail [Roche], and phosphatase inhibitor cocktail I [5 μM cantharidin, 5 nM microcystin-LR, 25 μM bromotetramisole oxalate; Calbiochem]). The lysates were rocked for 30 min at 4°C and clarified by centrifugation. Nuclear extracts were prepared as previously described (3). Protein concentrations were determined by the Bradford method (Bio-Rad).

Whole-cell extracts for assaying endogenous protein kinase activity were prepared as described in reference 33, by lysing cells in 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Nonidet P-40 (Sigma), protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail set I (Calbiochem) and clarifying them by centrifugation.

Sodium dodecyl sulfate (SDS) lysates were prepared by lysing cells with 2× SDS-polyacrylamide gel electrophoresis (PAGE) buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue, and 200 mM dithiothreitol [DTT]). Lysates were sonicated or vortexed to decrease viscosity, incubated in boiling water for 5 min, and clarified by centrifugation.

Phosphopeptide-specific antibody production. The phosphopeptide LSFS309p (LGEGNGpSPNHQPEC, where pS stands for phosphoserine), synthesized at Tufts University Core Facility, was coupled to keyhole limpet hemocyanin according to the manufacturer's instructions (Pierce Biotechnology, Inc.) and injected into New Zealand White rabbits (Covance). The immunoglobulin G fraction from total immunized rabbit serum was enriched using a HiTrap protein G column (GE Healthcare). Phosphopeptide-specific antibodies were purified by passage through SulfoLink (Pierce) columns coupled to the phosphopeptide and then to the corresponding unphosphorylated peptide.

Immunoblot analysis. Proteins were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (New England Nuclear). The membranes were blocked in 5% dry milk in TBS-T (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% Tween 20) and incubated with primary antibody overnight. Antibodies against LSF included anti-LSF (BD Biosciences), anti-LSF (314) (a rabbit polyclonal antibody raised against LSF amino acid residues 314 to 329 [33a]), anti-LSF pep1-2 (49), anti-S309p, and anti-S291p (11a). Other antibodies used were against the following: cyclin C (T-19) (Santa Cruz), cyclin C (BD Biosciences), FLAG M2 (Sigma), p44/42 mitogen-activated protein kinase (MAPK), pp44/42 MAPK (Thr202/Tyr204), pAkt (Ser 473), pp70 S6 kinase (Thr 421/Ser 424) (Cell Signaling Technology), CDK2 (Millipore), CDK2 (M2) (Santa Cruz), HA (Covance), and β-actin (Sigma). Blots were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Bio-Rad) and developed using a SuperSignal West Pico enhanced chemiluminescence kit (Pierce).

Immunoprecipitation. TD cell lysates from 10^7 cells were incubated with 2 μg anti-cyclin C antibody (T-19) (Santa Cruz) overnight at 4°C with rocking. Immune complexes were recovered on fast-flow protein A Sepharose beads (Upstate), washed four times with TD lysis buffer, and eluted using 2× SDS-PAGE loading buffer. Immunoprecipitation with FLAG-tagged proteins was performed with either 500 μg of TD cell lysates (for FLAG-tagged cyclin C and cyclin E) or 200 μg of nuclear extract (for FLAG-tagged LSF) from transfected 293T cells. Extracts were incubated at 4°C with 25 μl of anti-FLAG-M2 agarose beads (Sigma), preblocked with mock-transfected cell extract either overnight (FLAG-tagged cyclins) or for 1 h (FLAG-tagged LSF). Immune complexes were washed four or five times with TD lysis buffer or wash buffer (50 mM Tris-HCl, pH 7.5, 15 mM EGTA, 100 mM NaCl, 1% Triton X-100, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride) and eluted using 2× SDS-PAGE loading buffer.

In vitro kinase assays. Cyclin/CDK complexes were purified using glutathione Sepharose resin from cell lysates of Sf9 insect cells coinfected with baculoviruses encoding a cyclin (A, E, D1, D2, or D3) and a glutathione *S*-transferase (GST)-tagged kinase (CDK2, CDK4, or CDK6) (16). For in vitro kinase assays, 10 ng of purified kinase was incubated with either 1 μg recombinant, purified wild-type (WT) or substitution mutant His-LSF (49); 1 μg histone H1; or 1 μg GST-pRb-C (residues 792 to 928) (29) at 30°C for 30 min in 50 mM HEPES (pH 7.0), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 1 μM unlabeled ATP, and 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) in a final volume of 50 μl. Reactions were terminated by addition of 4× SDS-PAGE loading buffer. Samples were subjected to SDS-PAGE through a 7.5% polyacrylamide gel.

To assay cyclin C-associated kinase activity, 293T cells were transiently transfected with pCMV-Bam-Neo cyclin C FLAG, with or without pRcCMV-CDK3-HA or pRcCMV-CDK2, using Transit-LT1 (Mirus). At 48 hours post-transfection, cells were harvested. TD cell lysate (500 μg of total cell protein) was immunoprecipitated with 20 μl of anti-FLAG-M2 agarose beads (Sigma) and washed three times with TD lysis buffer and twice with kinase buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT, protease inhibitor cocktail [Roche], phosphatase inhibitor cocktail I [Calbiochem]). The beads were incubated at 30°C for 30 min with mixing in 20 μl of kinase buffer containing 20 μM unlabeled ATP, 10 μCi [γ-^{32}P]ATP (3,000 Ci/mmol) (Perkin Elmer), and 0.5 μg His-LSF. Reactions were terminated by addition of 4× SDS-PAGE loading buffer and subjected to SDS-PAGE through a 7.5% polyacrylamide gel.

GST-pRb (379-928) was purified as previously described (43) and used as the substrate to assay endogenous cyclin E- and cyclin C-associated kinase activity, as previously described (33).

Phosphoamino acid analysis and phosphopeptide mapping. For phosphoamino acid analysis, His-LSF substrates phosphorylated in vitro were sepa-

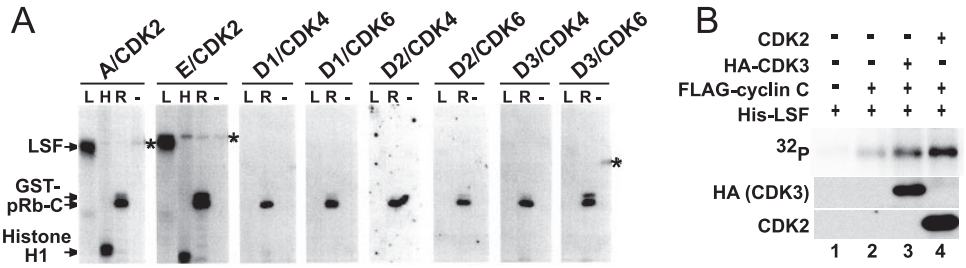

FIG. 1. G_1 cyclin complexes containing either CDK2 or CDK3, but not CDK4 or CDK6, phosphorylate LSF in vitro. (A) Purified CDK complexes, as indicated, were incubated in the presence of [γ-^{32}P]ATP with either His-LSF (L), a fusion protein between GST and a C-terminal fragment of pRb (R), histone H1 (H), or no exogenous substrate (−). Radiolabeled products were visualized by autoradiography after SDS-PAGE. Asterisks indicate products resulting from autophosphorylation. (B) FLAG-tagged cyclin C, either alone or with CDK2 or HA-tagged CDK3, was expressed in 293T cells, and exogenously expressed cyclin C was immunoprecipitated using anti-FLAG antibody. Products from in vitro kinase assays with His-LSF were separated by SDS-PAGE, transferred to a PVDF membrane, and visualized by autoradiography (^{32}P). The membrane was also immunoblotted using antibodies against CDK2 (M2) or the HA tag.

rated by 7.5% SDS-PAGE and then transferred to PVDF membranes (NEN Research Products). Membrane strips containing LSF were incubated in 100 μl 6 M HCl at 110°C for 60 min. The samples were prepared, and products containing roughly equivalent amounts of radioactivity were analyzed on thin-layer cellulose plates as previously described (7); products were detected using a phosphorimager.

For phosphopeptide mapping, phosphorylated WT or mutant His-LSF substrates were transferred to nitrocellulose membranes (Schleicher & Schuell). For chymotrypsin digestion, membranes were incubated in 0.5% polyvinylpyrrolidone in 100 mM acetic acid, rinsed, and incubated twice in ammonium bicarbonate with 10 μg chymotrypsin (sequencing grade; Boehringer Mannheim) for 2 h at 25°C. For endoproteinase Glu-C digestion, membranes were incubated twice in ammonium bicarbonate with 8 μg Glu-C (V8 protease, sequencing grade; Boehringer Mannheim) for a total of 2 h at 25°C. The samples were washed and lyophilized as described previously (7). Peptides were separated on thin-layer cellulose plates by electrophoresis at pH 1.9 (88% formic acid-acetic acid-H_2O [25:78:897]) for 25 min at 1.0 kV in the first dimension, followed by ascending chromatography in buffer (butanol-pyridine-acetic acid-H_2O [75:50:15:60]) for 8 h in the second dimension. Labeled peptides were detected using a phosphorimager.

Transient transfection and reporter assays. Exponentially growing NIH 3T3 cells were transfected using 2 μg plasmid DNA and 10 μl Lipofectamine (Invitrogen) per 35-mm plate. Cells were harvested at 36 to 48 h posttransfection with passive lysis buffer (Promega). Firefly luciferase and *Renilla* luciferase activities were measured using the dual luciferase assay system (Promega). The relative activation of the reporter construct was determined by dividing the firefly luciferase values by the *Renilla* luciferase values to normalize for transfection efficiency.

RNA interference. Short interfering RNAs (siRNAs) were transfected into 10^5 freshly seeded NIH 3T3 cells by using 10 μl Lipofectamine 2000 per 35-mm plate at a final siRNA concentration of 20 nM. The cells were incubated for 24 h and subsequently growth arrested and stimulated by addition of medium containing 10% calf serum. The siRNAs were double-stranded, 19-nucleotide RNAs containing two 3′ deoxyribosylthymine overhangs; siRNA against murine cyclin C (5′-GUUAUUGCUACUGCUACAG-3′) (Dharmacon) was designed by S. Ren (Dana-Farber Cancer Institute, Boston, MA), and the nonspecific siRNA was negative control 1 (catalog no. 4611; Ambion).

Retrovirus production and infection. Retroviruses were produced by transfecting 7×10^5 Phoenix Eco cells (either the parental vector or LSF S309A, cyclin C-FLAG, or CDK3-HA derivatives) with 2 μg LZRSpBMN-linker-IRES-EGFP and 0.5 μg pCL-Eco, using Transit-LT1 transfection reagent (Mirus). Viral supernatants were harvested at 48 and 72 h posttransfection. NIH 3T3 cells were infected with virus stocks in the presence of 8 μg/ml polybrene at the minimal concentration, resulting in >95% transduced cells, as estimated by the percentage of green fluorescent cells.

Quantitative reverse transcriptase PCR. Total RNA was prepared using Trizol reagent (Invitrogen) per the manufacturer's instructions. One microgram of RNA was reverse transcribed using random hexamers and MultiScribe reverse transcriptase (Applied Biosystems). The cDNAs were analyzed by quantitative PCR using SYBR green PCR master mix (Applied Biosystems) with an ABI Prism 7900 sequence detection system (Applied Biosystems). Threshold cycles for triplicate PCRs were determined using Sequence Detection System software (version 2.2.2; Applied Biosystems), and relative transcript abundance was determined by normalizing against β2-microglobulin mRNA. All primer sets generated unique products, as determined by dissociation curves, and amplified linearly, as determined by serial dilution of cDNA. Primer sets for quantitative PCR analysis included those for thymidylate synthase mRNA (*Tyms*) (TGCCA ATGGATCCCGAGAT and ACTCTGCTCCAAAATGCCTCC), cyclin E1 mRNA (*Ccne1*) (GCCATGCCAAGGGAGAGAG and GCCACATTTGCCTT CCTTTTT), MCM3 mRNA (*Mcm3*) (TACCAGAACAAGGTTCGGGAA and AGGCGGTTAGCCCTCTTTTC), and β2-microglobulin mRNA (*B2m*) (TTC AGTATGTTCGGCTTCCC and CCTGGTCTTTCTGGTGCTTG).

RESULTS

Multiple CDK complexes phosphorylate LSF in vitro. To determine whether G_1 CDKs were capable of phosphorylating LSF, we assayed a panel of purified cyclin/CDK complexes in vitro, including D-type cyclins in complex with either CDK4 or CDK6 and cyclin A or E in complex with CDK2 (Fig. 1A). Additional assays were also performed with cyclin C-associated complexes, exogenously expressed and immunoprecipitated from 293T cells (Fig. 1B). All the purified cyclin/CDK complexes were active, as demonstrated by phosphorylation of a C-terminal fragment of the retinoblastoma protein (pRb) (Fig. 1A, lanes R) or histone H1, included as a second positive control for CDK2 activity (Fig. 1A, lanes H). Of these kinases, only cyclin E/CDK2 and cyclin A/CDK2 phosphorylated purified, recombinant histidine-tagged LSF (His-LSF) (Fig. 1A, lanes L). Additionally, LSF was phosphorylated by a kinase in cyclin C immunoprecipitates even in the absence of exogenously expressed CDK (Fig. 1B, lane 2).

Whereas previous studies with human cell lines indicated that cyclin C associates with CDK3 in early G_1, most laboratory mouse strains harbor a premature stop codon in *Cdk3* (51). By amplification and sequencing of genomic DNA from NIH 3T3 cells, we demonstrated that this cell line, which provides a robust system for study of cell growth regulation and cell cycle progression, similarly contains mutated *Cdk3* (data not shown). In the absence of CDK3, it is likely that CDK2, the kinase most closely related to CDK3 (25, 26), can compensate by associating with cyclin C. To initially test this hypothesis, CDK3 and CDK2 were independently coexpressed with cyclin C in human cells, and the ability of cyclin C-associated kinases to phosphorylate LSF in cyclin C immunoprecipitates was monitored. Not only CDK3 but also CDK2 significantly en-

hanced the cyclin C-associated phosphorylation of LSF (2.5- and 3.2-fold, respectively) (Fig. 1B, lanes 3 and 4). Furthermore, not only exogenously expressed HA-CDK3 but also exogenously expressed CDK2 coimmunoprecipitated with cyclin C, as shown by immunoblotting (Fig. 1B). These findings confirm previous results indicating that CDK3 productively associates with cyclin C (33) and demonstrate that CDK2 can also associate with cyclin C to form an active kinase complex. Both of these cyclin C-associated kinases can utilize LSF as a substrate in vitro.

Serine 309 is a major site of phosphorylation on LSF by both cyclin E/CDK2 and cyclin C/CDK3. Cyclin/CDK complexes are proline-directed kinases, targeting either serine or threonine residues directly preceding a proline residue. The sequence of LSF contains six serine-proline dipeptides and two threonine-proline dipeptides (24, 42, 53). To identify the sites phosphorylated by cyclin/CDK complexes, we initially mapped sites phosphorylated by cyclin E/CDK2 in vitro, given the availability of high-purity enzyme and its ability to phosphorylate LSF with high efficiency. Phosphoamino acid analysis of in vitro-phosphorylated LSF (Fig. 2C, upper) indicated that serine residues were predominantly targeted, as the ratio of phosphoserine to phosphothreonine was 22.

By combination of phosphopeptide mapping of WT LSF and of alanine substitution mutants, all the sites for phosphorylation by cyclin E/CDK2 were identified. Two-dimensional analysis of WT His-LSF phosphorylated in vitro revealed four major radiolabeled chymotryptic peptide products (Fig. 2A, spots a, b, c, and ∗). For equivalent amounts of radiolabeled product from LSF S309A, spot "a" was absent (Fig. 2A), identifying this as the phosphopeptide containing residues 302 to 336. Both S289 and S291 are present in a single nine-residue chymotryptic product (residues 286 to 294). Comparison of the phosphopeptide patterns from the WT with those of the S289A, S291A, and S289A/S291A mutants led us to conclude that two distinctly migrating phosphopeptides resulted from this peptide, depending on whether S289 or S291 was phosphorylated (Fig. 2A, spots b and c). We hypothesize that the formation of a salt bridge between phosphorylated serine 291 and asparagine 287 alters the mobility of this peptide (7, 34). Finally, the chymotryptic map of phosphorylated LSF S289A/S291A/S309A lacked all four major phosphopeptide products, demonstrating that serines 289, 291, and 309 are the major cyclin E/CDK2 phosphorylation sites in LSF (Fig. 2A). We therefore concluded that the final product in the WT and mutant protein maps (spot ∗) represented a partial chymotryptic digestion product containing serines 289, 291, and 309.

Phosphopeptide mapping using a second protease, Glu-C (Fig. 2B), concurred with the identification of the phosphorylated serine residues. The phosphopeptide containing Ser 289 and Ser 291 phosphorylation sites is 22 amino acids, resulting in identical migrations of the phosphopeptide (spot e), no matter which serine was phosphorylated. This peptide was unlabeled only upon mutation of both Ser 289 and Ser 291. Because there is no additional product corresponding to a doubly phosphorylated phosphopeptide in the WT pattern (compared to that of the S289A or S291A mutant), we conclude that cyclin E/CDK2 phosphorylates LSF on either Ser 289 or Ser 291 but not simultaneously on both, perhaps due to steric hindrance. As with the chymotryptic phosphopeptide

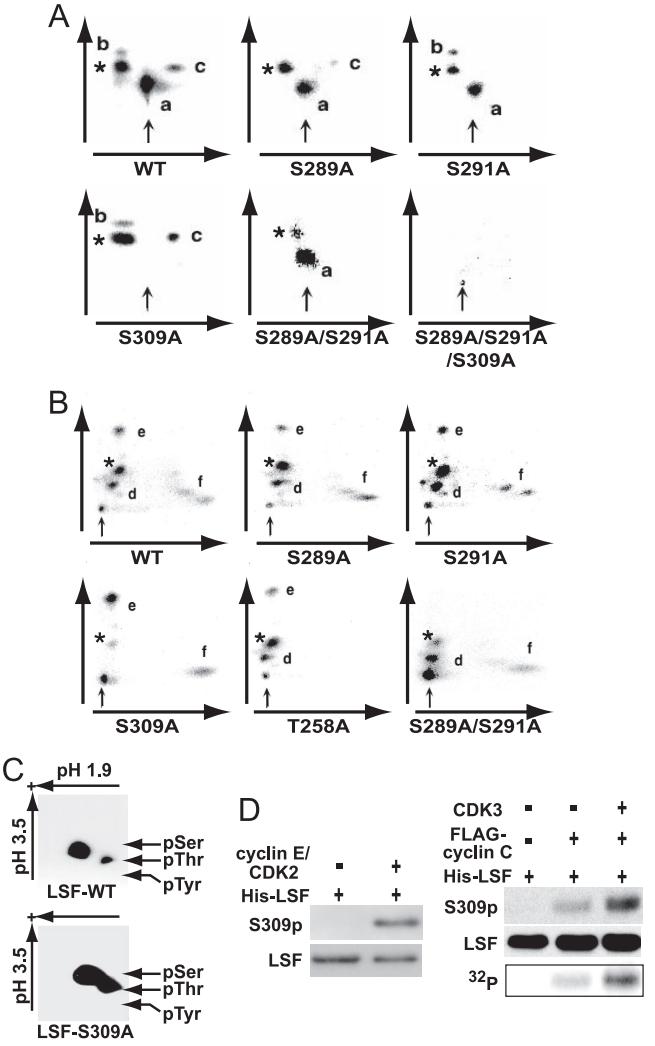

FIG. 2. Cyclin E/CDK2 and cyclin C/CDK both phosphorylate LSF on S309 in vitro. Chymotryptic (A) or Glu-C (B) phosphopeptide analysis of WT His-LSF and the indicated amino acid substitution mutants of His-LSF phosphorylated in vitro by cyclin E/CDK2. Samples were processed as described in Materials and Methods. The origins are indicated with arrows, and peptides are indicated with letters or an asterisk. The corresponding phosphopeptide sequences for panel A are VNNSPSPGF (S289 and S291) (a and b) and SLGEGNGSPN HQPEPPPPVTDNLLPTTTPQEAQQW (S309 and T329) (c), and those for panel B are GNGSPNHQPEPPPPVTDNLLPTTTPQE (S309 and T329) (d), ITVNNSPSPGFNSSHSSFSLGE (S289 and S291) (e), and KRTPHE (T258) (f). Electrophoresis is indicated by the rightward arrow and chromatography by the upward arrow. (C) Phosphoamino acid analysis of WT His-LSF and His-LSF S309A phosphorylated by cyclin E/CDK2 in vitro. Arrows mark the positions of phosphoamino acid standards (pSer, phosphoserine; pThr, phosphothreonine; and pTyr, phosphotyrosine). The direction of electrophoresis in each indicated buffer is shown on the respective axis. Roughly equivalent amounts of total radioactivity were loaded in each case. (D) His-LSF was phosphorylated in vitro either with purified cyclin E/CDK2 (left) or with immunoprecipitated cyclin C-associated kinase in the presence of radiolabeled ATP (right). Following SDS-PAGE, reaction products were either immunoblotted with anti-S309p and anti-LSF (314), as indicated, or autoradiographed (^{32}P).

mapping, one Glu-C phosphopeptide was absent in the S309A mutant (spot d). As before, spot * corresponded to a partial digestion product containing serines 289, 291, and 309.

LSF contains only two threonine-proline dipeptides, at T258 and T329. Chymotryptic digestion results in a lysine-rich, highly charged T258-containing peptide (+12, compared to neutral or ±1 for the other peptides), which would migrate to the far right in the electrophoretic dimension. Low-intensity candidate products in the chymotryptic phosphopeptide maps were difficult to resolve (data not shown). Cleavage instead with protease Glu-C permitted definitive identification of T258 (contained in a six-residue peptide) as the major site of threonine phosphorylation by cyclin E/CDK2 (Fig. 2B). In particular, comparison of the Glu-C phosphopeptide map of WT LSF with that of the T258A mutant revealed that the pair of spots "f" migrating to the far right corresponds to the short phosphopeptide containing Thr 258. Thr 329, the other potential threonine target, is located on the same peptide as Ser 309 after digestion with either chymotrypsin or Glu-C. The absence of doubly phosphorylated Ser 309-containing phosphopeptides and the lack of labeling of the Ser 309-containing peptides in the S309A mutant, combined with the positive identification of Thr 258 as a target of cyclin E/CDK2 phosphorylation, led to the conclusion that the only threonine phosphorylated by cyclin E/CDK2 is Thr 258.

The relative efficiencies of phosphorylation in vitro at the various serines in LSF were quantified more accurately by phosphoamino acid analysis of LSF mutants with substitutions at single residues. We note that similar amounts of radioactivity from both WT and mutant LSF products were applied to all thin-layer plates. Thus, in the case of LSF S309A (Fig. 2C, lower), where the degree of overall phosphorylation was greatly reduced (data not shown), a significantly larger molar amount of product was analyzed. The phosphoserine-to-phosphothreonine ratio for LSF S309A dropped to 3.9 (Fig. 2C, lower). Compared to the ratio of 22 for WT LSF, this represents a 5.6-fold decrease specifically in serine phosphorylation. In contrast, mutation of either S289 or S291 alone did not significantly alter the ratio of radiolabeled phosphoserine to phosphothreonine (data not shown). Taken together, these data indicate that S309 is the major site of in vitro phosphorylation in LSF for cyclin E/CDK2 in vitro.

To more effectively monitor phosphorylation at S309, we generated antibodies that specifically recognize LSF phosphorylated at this site (anti-S309p). As expected, the antibodies did not detect unphosphorylated WT His-LSF but did detect it once it was phosphorylated in vitro by cyclin E/CDK2 (Fig. 2D, left). Phosphorylation at S309 was similarly observed upon immunoblotting of LSF phosphorylated in vitro by immunoprecipitated cyclin C-associated kinase (Fig. 2D, right). Coexpression of CDK3 with cyclin C not only increased the amount of radiolabeled phosphate incorporated into LSF in the in vitro kinase assay, as observed previously (Fig. 1B), but also specifically enhanced phosphorylation of LSF at S309. Taken together, these data demonstrate that LSF is phosphorylated on S309 by both cyclin E/CDK2 and cyclin C-associated kinases in vitro.

LSF is phosphorylated on S309 in early G_1. To investigate the kinetics of LSF phosphorylation at S309 throughout G_1, from quiescence through S phase, NIH 3T3 cells were growth arrested and stimulated to enter the cell cycle. The synchrony

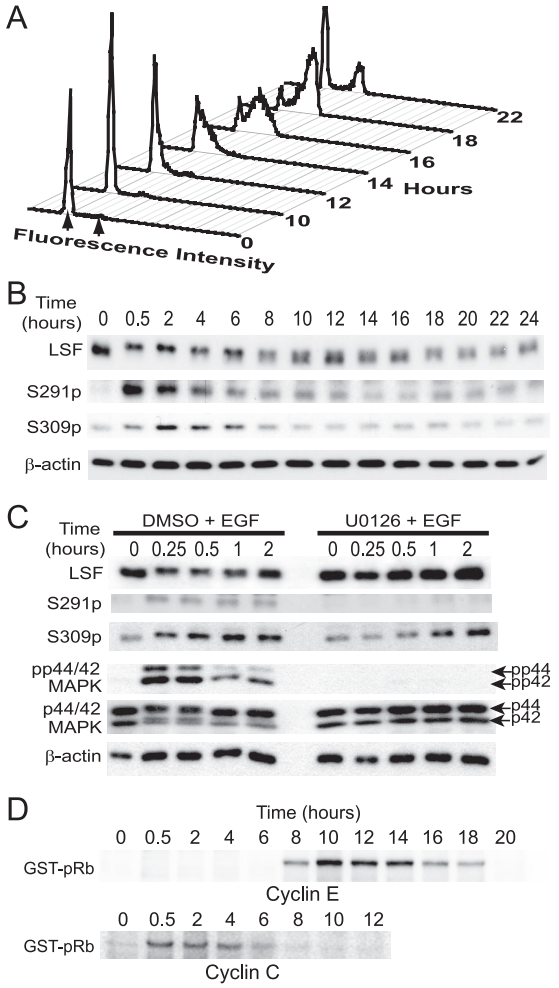

FIG. 3. LSF is phosphorylated with differing kinetics in early G_1 at S291 and S309. (A) Growth-arrested NIH 3T3 cells were stimulated to reenter the cell cycle by addition of 10% serum. At the indicated times before (zero hour) or after mitogenic stimulation, the DNA content of the cells was analyzed by propidium iodide staining and flow cytometry. The peaks of fluorescence intensity that correspond to G_0/G_1 and G_2 DNA contents are indicated by arrows. (B) Total cellular extracts from a batch of cells parallel to those in panel A were prepared in TD buffer at the indicated time points. The extracts were analyzed by immunoblotting with antibodies against LSF (BD), S291p, S309p, and β-actin. (C) Growth-arrested NIH 3T3 cells were pretreated, prior to stimulation with EGF, with either U0126 or dimethyl sulfoxide (vehicle). TD lysates prepared at the indicated time points were analyzed by immunoblotting with antibodies against LSF (BD), S291p, S309p, p44/42 MAPK, pp44/42 MAPK (Thr202/Tyr204), and β-actin. The apparent decreases in LSF levels at the early time points shown in control lanes (top left) are a consequence of interference of S291 phosphorylation with recognition of LSF by this commercial LSF monoclonal antibody (data not shown). (D) Growth-arrested NIH 3T3 cells were stimulated with 10% calf serum. Whole-cell extracts prepared at the indicated time points were immunoprecipitated using antibodies against cyclin E or cyclin C, as indicated. The immunoprecipitates were assayed for kinase activity by using radiolabeled ATP and GST-pRb (379-928) as a substrate. Products were resolved by SDS-PAGE, transferred to a PVDF membrane, and visualized with a phosphorimager.

of the cells was monitored by staining with propidium iodide and analyzing the DNA content using flow cytometry (Fig. 3A). Following serum deprivation for 36 h, more than 96% of the cells contain levels of DNA consistent with G_0. Cells entering S phase were initially detected at 12 h poststimulation,

and cells in G_2/M were observed by 18 h poststimulation. Maximally, 7% of cells remain in G_0/G_1 at this point.

LSF protein levels did not appreciably change between G_0 and S phases (Fig. 3B, top row), although the mobility of the protein shifted immediately after serum stimulation, as previously observed. The mobility shift reflects phosphorylation on S291, which we have previously shown is dependent on ERK activity (30, 49). With a phosphopeptide-specific antibody (anti-S291p), S291 phosphorylation was not detectable in quiescent cells (Fig. 3B, second row). Thirty minutes after growth stimulation, S291 phosphorylation was maximal, and the level decreased as cells progressed through G_1, consistent with LSF mobility changes. In contrast, LSF was detectably, although minimally, phosphorylated on S309 in quiescent fibroblasts. Phosphorylation at this site increased by 2 hours poststimulation, once again decreasing later in G_1 (Fig. 3B, third row). Overall, both S291 and S309 phosphorylations are inducibly phosphorylated in early G_1, but the kinetics of modification are temporally distinct. Notably, phosphorylations at both S291 and S309 decrease substantially between 8 and 12 h after stimulation, prior to the G_1/S transition.

Given the early G_1 phosphorylation of LSF on S309 and the ability of ERK to phosphorylate LSF on S309 (as well as S291) in vitro (data not shown), we tested whether phosphorylation at S309 in early G_1 might also be dependent on MEK/ERK signaling in vivo. Quiescent NIH 3T3 cells were treated with the MEK 1/2 inhibitor U0126 prior to mitogenic stimulation with EGF. As monitored by immunoblotting for phosphorylated ERK (pp44/42 MAPK), EGF treatment robustly activated ERK (Fig. 3C, left). Furthermore, as anticipated, pretreatment with U0126 completely blocked ERK activation (Fig. 3C, right). Inhibition of MEK also completely blocked phosphorylation of LSF at S291 (Fig. 3C), demonstrated by both the lack of reactivity to anti-S291p and the lack of a mobility shift in LSF, consistent with our previous findings (30). In contrast, MEK inhibition did not block the maximal level of phosphorylation of LSF at S309 (Fig. 3C), demonstrating that the MEK/ERK pathway is not required for this modification in response to growth signaling. However, we noted that pretreatment with U0126 did reproducibly decrease S309 phosphorylation at 15 and 30 min poststimulation. Thus, S309 may be targeted to some degree by ERK immediately after serum stimulation, but this does not account for the bulk of phosphorylation in early G_1.

To assess whether cyclin C/CDK2 and cyclin E/CDK2, instead, might be candidate kinases for LSF S309 phosphorylation in early G_1, we assayed the kinetics of activation of these kinases following growth factor stimulation (Fig. 3D). As expected (33), cyclin C-associated kinase activity was detected within the first hour following growth factor stimulation, consistent with the kinetics of LSF phosphorylation. In contrast, cyclin E-associated kinase activity, as anticipated (15, 22, 33), was not detected until late G_1, 8 to 10 h after cell stimulation. These results are consistent with LSF S309 phosphorylation by cyclin C but not cyclin E complexes.

Exogenously expressed LSF robustly associates with cyclin C, with phosphorylation at S309 being inhibited by p21 and p27. To determine whether LSF may be a substrate for cyclin C-containing complexes in vivo, we first examined whether LSF associates in vivo with cyclin C. Many substrates targeted by CDK2 specifically interact with the cyclin-kinase complex (1, 9). LSF was transiently expressed in asynchronously growing human cells, along with FLAG-tagged cyclin C or FLAG-tagged cyclin E as a control and extracts immunoprecipitated with anti-FLAG antibodies. Similar levels of the two cyclins were immunoprecipitated (Fig. 4A, bottom). At these highly expressed levels, LSF robustly coimmunoprecipitated with FLAG-cyclin C (Fig. 4A, lane 2) but only weakly associated with FLAG-cyclin E (Fig. 4A, lane 3). These results support the hypothesis that LSF may be a biological target of a cyclin C-associated kinase.

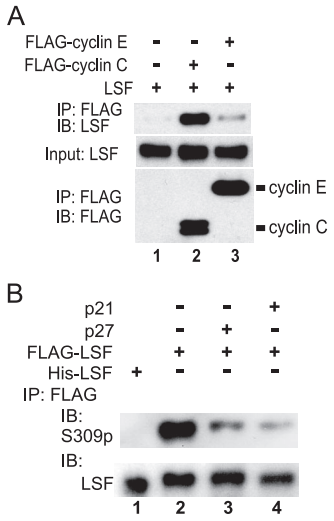

FIG. 4. LSF associates with cyclin C and is phosphorylated by CDKs in asynchronous human cell lines. (A) LSF associates more strongly with cyclin C than with cyclin E. 293T cells were transfected with pEF1α-LSF alone or in combination with either FLAG-tagged cyclin C or FLAG-tagged cyclin E. Cellular extracts, made in TD buffer, were immunoprecipitated (IP) with antibody against FLAG, and the precipitates were immunoblotted (IB) with antibodies against LSF (314) and FLAG, as indicated. The input extract (1% of the amount immunoprecipitated) was also immunoblotted with anti-LSF (314); the two anti-LSF immunoblots represent equivalent times of exposure. (B) CDK2/3 inhibitors reduce S309 phosphorylation in vivo. Results are shown for immunoblot analysis of FLAG-LSF immunoprecipitated from 293T cells in which the FLAG-LSF was expressed either alone or in combination with the CDK inhibitors p21 or p27. The blots were sequentially probed with anti-S309p and anti-LSF pep1-2. His-LSF was loaded onto the gel as a marker (lane 1).

To address whether such association between LSF and cyclin C might result in phosphorylation of LSF at S309 in vivo, the CDK inhibitors p21 and p27 were transiently expressed along with FLAG-tagged LSF in these asynchronously growing human cells. The exogenously expressed LSF was then immunoprecipitated and immunoblotted for both total LSF and phosphorylation at S309. Expression of both CDK inhibitors substantially diminished the degree of S309 phosphorylation (Fig. 4B). Both p21 and p27 can associate not only with CDK2 (40) but also with CDK3 to inhibit its activity (8, 17). Therefore, these results are consistent with targeting of LSF at S309 by either a CDK2 or a CDK3 complex in vivo.

Endogenous cyclin C specifically associates with LSF in early G_1 and is required for concomitant phosphorylation of LSF on S309. To determine whether interaction of LSF with cyclin C is biologically relevant, especially with regard to the regulated phosphorylation of LSF during cell cycle progression

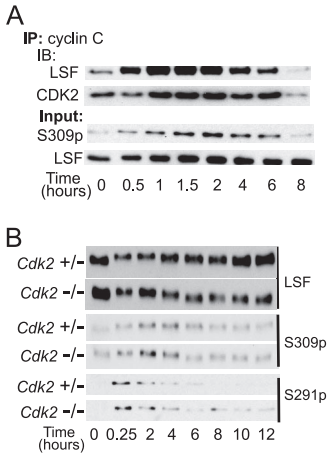

FIG. 5. Association of cyclin C/CDK2 with LSF in early G_1 and plasticity of LSF phospharylation by CDKs. (A) Quiescent NIH 3T3 cells were mitogenically stimulated and extracts prepared at the indicated time points. Extracts immunoprecipitated (IP) with antibodies against cyclin C were then immunoblotted (IB) with antibodies against LSF (BD) and CDK2. Input extracts (2% of the amount immunoprecipitated) were also immunoblotted with antibodies against LSF (BD) or S309p, as indicated. (B) CDK2 is not essential for S309 phosphorylation in early G_1. MEFs derived from *Cdk2*$^{-/-}$ or *Cdk2*$^{+/-}$ embryos were growth arrested and then stimulated with 10% serum. Extracts prepared in TD lysis buffer at various time points after stimulation were analyzed by immunoblotting with anti-LSF (BD), anti-S291p, and anti-S309p.

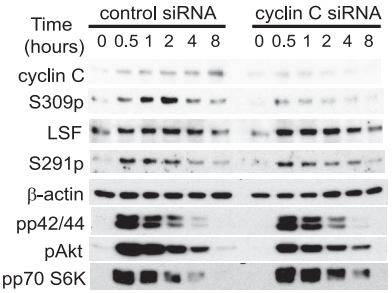

FIG. 6. Cyclin C-associated kinase phosphorylates LSF in early G_1 in growth-regulated mouse fibroblasts. NIH 3T3 cells were transfected with either nonspecific siRNA (control siRNA) or siRNA against cyclin C, growth arrested, and stimulated for the indicated amounts of time with serum. Extracts made in TD lysis buffer were immunoblotted with antibodies against cyclin C (BD Biosciences), S309p, S291p, LSF (314), β-actin, pp44/42 MAPK (Thr202/Tyr204), pAkt (Ser 473), and pp70 S6 kinase (Thr 421/Ser 424).

following exit from quiescence, endogenous cyclin C was immunoprecipitated from synchronized NIH 3T3 cell extracts and the precipitates were immunoblotted for levels of endogenous LSF (Fig. 5A, top row). LSF associated with cyclin C only weakly in quiescent cells, but the levels of complex between endogenous LSF and endogenous cyclin C increased upon mitogenic stimulation of quiescent NIH 3T3 cells, peaking 1 to 2 h after stimulation. Subsequently, the degree of interaction decreased to background levels by 8 h poststimulation (Fig. 5A, top row). Throughout this time course, LSF protein levels did not change significantly (Fig. 5A, bottom row).

Since the NIH 3T3 cells used in these experiments do not express functional CDK3, we tested whether cyclin C complexed instead with CDK2 in early G_1. Immunoblotting with antibody against CDK2 demonstrated robust coimmunoprecipitation with cyclin C, with the complex peaking 1 to 2 h after stimulation of the quiescent cells with serum (Fig. 5A, second row). Finally, as observed previously (Fig. 3B), LSF phosphorylation on S309 was elevated within 30 min and increased from 1 to 2 h after stimulation (Fig. 5A, third row), consistent with the kinetics of association of LSF with cyclin C. The coincidence between cyclin C/CDK2 complex formation and phosphorylation of LSF at S309 strongly suggests that cyclin C/CDK2 is the LSF S309 kinase in these cells. Furthermore, these data support the premise that CDK2 compensates for the lack of CDK3 activity in forming active complexes with cyclin C after cells exit G_0 into G_1.

The extent of plasticity in CDKs has been widely demonstrated, especially by the ability of CDK1 to suffice for cell cycle progression in the absence of all the G_1 CDKs (37). Thus, we tested whether, in the absence of both CDK3 and CDK2, LSF would still be phosphorylated on S309 in early G_1. Indeed, this phosphorylation occurred with normal kinetics when CDK2 knockout MEFs were growth arrested and restimulated to enter the cell cycle (Fig. 5B). This suggests that not only CDK2 but also CDK1 can productively associate with cyclin C in early G_1, although further studies are required to prove this point.

To establish definitively whether cyclin C-associated kinase activity is required for LSF S309 phosphorylation, cyclin C expression in NIH 3T3 cells was suppressed by siRNA. Following growth arrest and stimulation with serum, LSF phosphorylation on S309 and S291 was monitored by immunoblotting of cellular extracts (Fig. 6). Transfection with the cyclin C-specific siRNA, but not the control, nonspecific siRNA, efficiently suppressed cyclin C expression (Fig. 6, top row). The kinetics and extent of S309 phosphorylation on LSF in early G_1 were maintained in the presence of control siRNA but were severely diminished in the presence of cyclin C-specific siRNA (Fig. 6, second row). In contrast, ERK-mediated S291 phosphorylation and LSF protein levels were unaffected. Thus, cyclin C is specifically required for early G_1 phosphorylation of LSF on S309, peaking around 1 to 2 h after serum stimulation. The residual, low level of phosphorylation on S309 in the presence of siRNA targeting cyclin C occurred with early kinetics similar to those of S291 phosphorylation, suggesting that this may be mediated by ERK.

Suppression of cyclin C expression was previously reported to lead to a delay in reentry into the cell cycle from the G_0 phase (33). Although the assay used in that report measured largely rRNA synthesis, which is directly affected by pRb (a substrate of cyclin C/CDK3), we considered the possibility that a general delay in exit from G_0 induced by RNA interference suppression of cyclin C resulted in inhibition of other protein kinases that might phosphorylate LSF. We therefore determined whether several of the major signal transduction pathways activated upon entry into the cell cycle from quiescence were affected by cyclin C ablation. In particular, activation of MAPKs (ERK1 and -2), Akt, and p70 S6 kinase were analyzed by immunoblotting with appropriate antibodies. None of these induction profiles was significantly altered by the siRNA ablation of cyclin C (Fig. 6), demonstrating that quiescent cells with

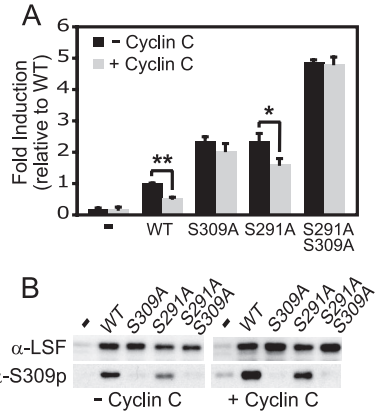

FIG. 7. Cyclin C expression reduces transactivation by LSF. (A) NIH 3T3 cells were cotransfected with 500 ng of either pCMV-LSF WT or the indicated alanine mutants of pCMV-LSF; 100 ng of the reporter plasmid pGL3B-WT4E1b, containing four LSF binding sites driving expression of firefly luciferase; 500 ng of an expression construct for cyclin C, when indicated; and phRL-TK, containing a herpes simplex virus-thymidine kinase promoter driving *Renilla* luciferase, to normalize for transfection efficiency. Expression from the reporter construct in the presence of each mutant is normalized to the degree of activation by WT LSF. The level of activation by WT LSF ranged between 5- and 10-fold. Error bars represent standard errors of the means from three independent experiments. By a pairwise *t* test, *P* values were <0.001 (**) or <0.05 (*), as indicated. By the Wilcoxon rank test, both of the indicated comparisons have *P* values of <0.05. (B) Immunoblot analysis, using antibodies against LSF (314) (α-LSF) and S309p, of extracts prepared in passive lysis buffer obtained from a representative of the experiment described for panel A.

severely decreased levels of cyclin C are not blocked at the G_0/G_1 transition but still respond to growth signals by activating major signaling pathways.

Cyclin C expression reduces the transactivation potential of LSF. Regulated phosphorylation of S309 in early G_1 suggests that this modification alters LSF activity. To test this hypothesis, we first transiently transfected NIH 3T3 cells with a synthetic LSF reporter construct containing four LSF binding sites upstream of a TATA sequence driving the firefly luciferase gene along with expression vectors for WT LSF, an LSF mutant with an alanine substitution at S309 or S291, or a double mutant at both residues. Mutating either S309 or S291 to alanine resulted in approximately 2.5-fold higher reporter activity than that of WT LSF (Fig. 7A, black bars). Significantly, the double mutant with alanine substitutions at S291 and S309 was approximately fivefold more active than WT LSF. WT and mutant LSF proteins are equivalently expressed in these experiments (Fig. 7B, left lanes). These data suggest that phosphorylations at S291 and S309 each inhibit the transactivation potential of LSF, although likely not by the same molecular mechanism, given the additive nature of their effects.

Consistent with the hypothesis that S309 phosphorylation limits the degree of transactivation by LSF, coexpression of cyclin C decreased LSF activity by 60% (Fig. 7A, gray bars), accompanied by a 2.0-fold increase in S309 phosphorylation (Fig. 7B, compare right lanes to left lanes). Cyclin C also reduced LSF S291A activity by 30%, with a 1.6-fold increase in phosphorylation at S309. Inhibitions of both WT and S291A LSF transcriptional activities were statistically significant but undoubtedly a minimal estimate of the degree to which dephosphorylation affects LSF transactivation, as both proteins were already significantly phosphorylated on S309 in the absence of cyclin C coexpression. These data indicate that LSF phosphorylation inhibits but does not completely abolish LSF transactivation. Instead of being an on/off switch, phosphorylation appears to modulate transcriptional potential. Notably, cyclin C expression did not significantly inhibit transactivation from LSF S309A or LSF S291A/S309A (Fig. 7A, gray bars).

Finally, a consistent observation was that mutation of S291 to alanine resulted in diminished levels of S309 phosphorylation (Fig. 7B). This suggests that there is cross talk between phosphorylation at these two sites (see further analysis in Discussion).

Phosphorylation of LSF on S309 in G_1 diminishes induction of thymidylate synthase gene expression in late G_1. We hypothesized that the downmodulation of LSF activity in early G_1 via phosphorylation provides a mechanism for preventing premature induction of its S-phase target gene *Tyms*. If this were the case, maintenance of phosphorylation on S309 should inhibit induction of *Tyms* by LSF at the G_1/S transition. We tested this hypothesis by investigating the induction of multiple G_1/S-regulated genes in the presence of elevated levels of cyclin C and CDK3, as assayed with NIH 3T3 cells transduced by retroviruses. As anticipated, cells transduced with control, parental retrovirus demonstrated induction of endogenous *Tyms* mRNA between 8 and 10 h following serum stimulation, with levels peaking between 14 and 16 h after mitogenic stimulation (Fig. 8A). However, in cells overexpressing cyclin C and CDK3, *Tyms* mRNA induction was reproducibly diminished, roughly twofold, at 12 and 14 h (Fig. 8B). To test whether cyclin C/CDK3 overexpression caused a nonspecific delay in G_1/S gene induction, the mRNA levels of the E2F3 target genes encoding cyclin E1 (*Ccne1*) and MCM3 (*Mcm3*) (19, 45) were also analyzed. In contrast to the diminished expression of *Tyms* mRNA, the expression of these two genes was unaffected (Fig. 8B and C).

Finally, to test whether the decrease in *Tyms* induction was mediated through LSF, LSF S309A, which cannot be phosphorylated by cyclin C/CDK, was concomitantly expressed. This mutant LSF protein completely rescued the inhibition of *Tyms* mRNA induction caused by cyclin C/CDK3 overexpression (Fig. 8A). Expression of LSF S309A did not alter induction of mRNAs encoding cyclin E1 and MCM3, however (Fig. 8B and C). These results support the hypothesis that the early-G_1 downregulation of LSF activity is a mechanism for preventing inappropriate induction of G_1/S target genes by LSF. More specifically, dephosphorylation of LSF on S309 is necessary, although not sufficient (given that *Tyms* was not prematurely induced during G_1), for efficient activation of an LSF target gene(s) at the G_1/S transition. These data also highlight that E2F3 activation of G_1/S genes and LSF activation of at least one G_1/S gene occur by parallel pathways.

DISCUSSION

The transcription factor LSF is required for cell cycle progression from quiescence into S phase (11, 31). Previous studies regarding regulation of LSF following mitogenic stimulation of cells identified multiple MAPK signal transduction

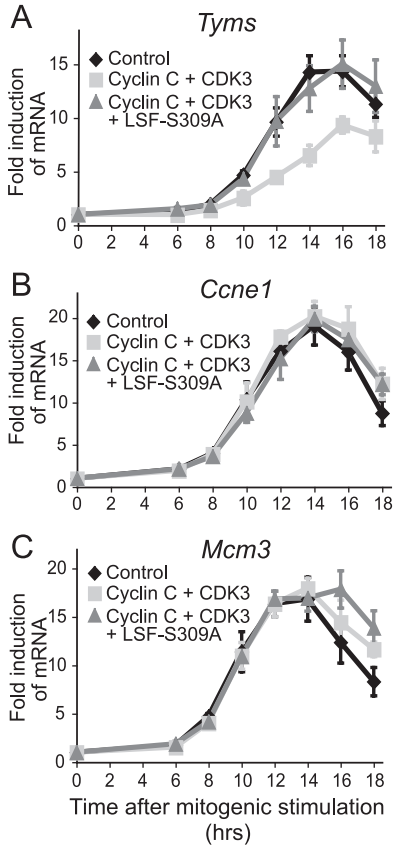

FIG. 8. Cyclin C/CDK3 overexpression decreases induction of *Tyms* at the G_1/S transition. NIH 3T3 cells were infected with either the parental retrovirus (control) or viruses overexpressing cyclin C and CDK3, with or without a virus overexpressing LSF S309A. Cells were growth arrested by serum deprivation and then stimulated to reenter the cell cycle by addition of 10% serum. Total cellular RNA was prepared at the indicated time points and analyzed by quantitative reverse transcriptase PCR for expression of the genes encoding thymidylate synthase (*Tyms*) (A), cyclin E1 (*Ccne1*) (B), and MCM3 (*Mcm3*) (C). Results shown are averages for four independent experiments using cells transduced with two separate preparations of retroviruses. Error bars indicate the standard errors of the means.

pathways, critical for progression of cells into the cell cycle, that directly target LSF on S291 in different cell types (30, 47, 49, 52). Here, we demonstrate that another kinase activity involved in G_0 exit in fibroblasts, cyclin C-associated CDK (33), also directly targets LSF (on S309) shortly after mitogenic stimulation of quiescent mouse fibroblasts. The targeted modification of S309 alters the transcriptional potential of LSF, as activation by WT LSF, but not LSF S309A, is diminished by coexpression of cyclin C. Additionally, overexpression of cyclin C and CDK3 inhibits the late-G_1/S-phase induction of the LSF target gene *Tyms.* This effect is completely rescued by coexpression of the nonphosphorylatable S309A mutant of LSF. Thus, phosphorylation of S309 by cyclin C-associated CDK in early G_1 appears to downmodulate LSF as a means for preventing induction of S-phase target genes prematurely. It is noteworthy that phosphorylation of LSF did not affect expression of the E2F target genes encoding cyclin E1 and MCM3, indicating that LSF and E2F3 act in parallel pathways to regulate distinct target genes at the G_1/S transition. Significantly, other than pRb (33), LSF is the only known substrate of cyclin C-associated CDK that is active in early G_1 following reentry of quiescent cells into the cell cycle.

Phosphorylation of LSF on S309 by cyclin C/CDK complexes was demonstrated by in vitro kinase assays, by coimmunoprecipitation of endogenous LSF and cyclin C in early G_1, and by abrogation of LSF phosphorylation on S309 following suppression of cyclin C with siRNA. Although LSF was also phosphorylated in vitro by cyclin E/CDK complexes, cyclin E/CDK activity was not detected until late G_1, as previously reported (15, 22, 33), several hours after LSF phosphorylation. In contrast, the kinetics of cyclin C/CDK activation in early G_1 were coincident with phosphorylation of LSF on S309.

Cyclin C is one of the smallest proteins in the cyclin family and contains only the N-terminal (not the C-terminal) cyclin box. It is in a distinct subfamily of cyclins from those associated with cell cycle control (e.g., cyclins A, B, D, and E) and is most similar to cyclin H (26). Cyclin C has largely been characterized as part of a cell cycle-independent complex with CDK8; this complex phosphorylates the C-terminal tail of RNA polymerase II (23). Association of cyclin C with another kinase, CDK3, was only recently demonstrated and characterized as a complex activated upon stimulation of quiescent cells to exit from G_0 (33).

Decreasing of cyclin C levels by siRNA was reported to delay exit from G_0, as assayed by the decrease in the ratio of cellular DNA to total cellular RNA, which reflects the dramatic increase in rRNA synthesis as cells are stimulated to reenter the cell cycle (33). Importantly, the polymerases responsible for rRNA synthesis, RNA polymerases I and III, are both inhibited by pRb (10, 50), which itself is targeted by cyclin C/CDK3 at the G_0/G_1 transition, apparently to relieve this inhibition (33). We found that ablation of cyclin C by siRNA did not affect the activation of other growth factor-regulated kinases (Akt, ERK, and p70 S6 kinase), indicating that cyclin C is required for certain aspects of the G_0/G_1 transition but not others. These results indicate that the decrease in phosphorylation of LSF at S309 upon diminishment of cyclin C levels by siRNA is not due to a general block to cell cycle reentry and suggest that phosphorylation of LSF represents a second role for cyclin C, beyond induction of rRNA, in cell cycle progression.

In the initial report of early G_1 cyclin C/CDK activity in a human cell line, the kinase that complexed with cyclin C was CDK3 (33). However, most laboratory mouse strains (51), as well as the NIH 3T3 cell line, lack functional CDK3 protein. The cell cycle-regulated CDKs exhibit an extraordinary capacity for compensation, as demonstrated by knockout mouse models. When necessary for survival, there is high plasticity of cyclin/CDK association, such that in the absence of their cognate CDK partner, cyclins can productively associate, although less efficiently, with noncognate CDKs (2, 27, 37). In that light, we hypothesized that in the absence of CDK3, cyclin C would likely associate with a replacement CDK in early G_1. CDK2, the kinase most closely related to CDK3 (26, 28), was the most obvious candidate. Our expectation was validated by the abilities of CDK2, like CDK3, to immunoprecipitate with cyclin C from cellular extracts and to enhance phosphorylation of LSF in vitro in cyclin C immunoprecipitates (Fig. 1B). Importantly, in the context of NIH 3T3 cells, CDK2 coimmunoprecipitated

with cyclin C in early G_1 (Fig. 5A), with kinetics similar to those previously demonstrated for the human T98G cell line for association of cyclin C with CDK3 (33).

The plasticity in the abilities of CDKs to complement each other has been best emphasized by the demonstrations that CDK1 is sufficient for cell cycle progression in the absence of all the G_1 CDKs (37) and that CDK1 can associate with cyclin D or cyclin E when CDK4/6 or CDK2, respectively, is absent (2, 37). CDK1 is the next-nearest family member relative to CDK3 and CDK2 (26, 28). Notably, LSF is still phosphorylated on S309 in early G_1 in MEFs lacking both CDK2 and CDK3 (Fig. 5B), suggesting that not only CDK2 but also CDK1 can productively associate with cyclin C in early G_1. This would be consistent with the ability of CDK1 to drive cell cycle progression through G_1 in the absence of all other G_1 CDKs (37).

Finally, although we demonstrated that cyclin C-associated CDK is the major kinase targeting S309 of LSF in early G_1, our results indicate that ERK may also contribute, either directly or indirectly, within the first hour after reentry into the cell cycle. Inhibition of MEK activity during serum stimulation of quiescent cells did not reduce S309 phosphorylation overall; however, the kinetics of phosphorylation was delayed (Fig. 3C). Furthermore, when cyclin C protein levels were diminished by siRNA treatment, the residual phosphorylation on S309 occurred within the first half hour after serum stimulation (Fig. 6).

LSF is a member of a small family of transcription factors that bind DNA as homotetramers and whose three-dimensional structures have not yet been solved. Nonetheless, regions of DNA interaction and oligomerization, which are highly conserved through evolution, have been mapped (47). Both S309 and S291 lie in a linker region in between the conserved DNA interaction and oligomerization regions (48). On the basis of the phenotypes of the nonphosphorylatable mutants with alanine substitutions at one or both of these residues (Fig. 7A), phosphorylation at either S309 or S291 inhibits LSF transactivation. The inhibition of LSF transactivation by phosphorylation at S309 was supported by the decrease in LSF function upon coexpression of cyclin C, which was accompanied by enhanced S309 phosphorylation (Fig. 7). The additive nature of the S291A and S309A mutants on LSF activity suggests that the two residues are likely to function in independent pathways. Phosphorylation by ERK at S291 can inhibit LSF DNA-binding activity, with the outcome being dependent on cell type and promoter sequence (30, 49, 52). In contrast, phosphorylation at S309 does not affect in vitro binding of LSF to a high-affinity binding site (R. Cacioppo and U. Hansen, unpublished observations). An alternative explanation for the effects of S309 phosphorylation is that it alters binding of LSF to coregulatory proteins, possibly through changes in conformation of LSF. The degree to which S291 or S309 phosphorylation regulates LSF function is likely to depend on the specific promoter/enhancer in question. We note that neither modification abolishes LSF function; each just diminishes activity. Teasing apart the functions of S291 and S309 phosphorylation in vivo is also complicated by the observation that mutants preventing phosphorylation at S291 (e.g., LSF S291A) also exhibit diminished phosphorylation at S309 (e.g., Fig. 7). Although not an absolute effect, it suggests that there is some degree of priming in which S291 phosphorylation (by ERK) enhances the suitability of S309 as a substrate for cyclin C/CDK2.

That LSF is required for productive cell cycle progression was initially revealed by our finding that disruption of LSF function prevented *Tyms* induction at the G_1/S boundary and induced apoptosis in S phase in both murine fibroblasts and human prostate cancer cells (31). In the present study, we show that cell cycle regulation of gene expression by LSF is not due to altered levels of total cellular LSF (Fig. 3B). Similarly, levels of LSF in nuclear extracts remain constant, with LSF being constitutively nuclear during progression of cells from G_0 into S phase (data not shown). However, this and previous studies describe extensive regulation of LSF modification, following exit of cells from quiescence, in early G_1. ERK-mediated phosphorylation of LSF at S291 occurs quantitatively and extremely rapidly (Fig. 3) (30, 49), followed by phosphorylation by cyclin C/CDK at S309. The phosphorylations of LSF at these two sites, mediated by ERK and cyclin C/CDK in early G_1, occur with identical kinetics in primary mouse embryo fibroblasts (Fig. 5B). We note that both of these phosphorylation sites are conserved in the LSF paralog LBP-1a as well as in orthologs of other species (48), suggesting that multiple LSF family members are similarly regulated in response to mitogenic stimuli.

In considering the biological roles of phosphorylation of LSF, it is important to recognize that both S291 and S309 are phosphorylated in early G_1 and then dephosphorylated in late G_1 (Fig. 3B). That phosphorylation at both sites inhibits LSF transactivation is thus consistent with the dephosphorylation of both sites prior to the G_1/S transition, when LSF activates *Tyms* gene expression. What is the biological role for such highly regulated phosphorylation at these two sites, considering that they are only dephosphorylated again during G_1 progression? Given that overexpression of cyclin C and CDK3 leads to a decrease in *Tyms* induction, which is rescued by coexpression of the nonphosphorylatable S309A mutant of LSF, we propose that the downmodulation of LSF by targeted phosphorylation provides a mechanism for limiting premature activation of G_1/S-regulated genes in response to an only temporary exposure to mitogenic cell signals. Such a mechanism ensures that such genes are activated only once the cell becomes committed to cell cycle progression.

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