Critical events occur during the G1 phase of the cell cycle that determine whether a cell will continue proliferating or will withdraw from the cell cycle and undergo differentiation or apoptosis (1). Transcription factors play a key role in these events, and their activity can be modified during G1 by a variety of intracellular and extracellular signals that alter their amount or their ability to interact with other proteins or with DNA (2).

MEF, a recently identified member of the E74 family of ETS-related transcription factors, is a strong transcriptional activator of cytokine gene expression. Using a green fluorescent protein gene reporter plasmid regulated by an MEF-responsive promoter, we determined that the transcriptional activity of MEF is largely restricted to the G1 phase of the cell cycle. MEF-dependent transcription was suppressed by the expression of cyclin A but not by cyclin D or cyclin E. This effect was due to the kinase activity generated by cyclin A expression, as co-expression of the cyclin-dependent kinase inhibitors p21 or p27, or a dominant negative form of CDK2 (DNK2), abrogated the reduction of MEF transcriptional activity by cyclin A. Cyclin A-CDK2 phosphorylated MEF protein in vitro more efficiently than cyclin D-CDK4 or cyclin E-CDK2, and phosphorylation of MEF by cyclin A-CDK2 reduced its ability to bind DNA. We determined one site of phosphorylation by cyclin A-CDK2 at the C terminus of MEF, using mass-spectrometry; mutation of three serine or threonine residues in this region significantly reduced phosphorylation of MEF by cyclin A and reduced cyclin A-mediated suppression of its transactivating activity. These amino acid substitutions also reduced the restriction of MEF activity to G1.

Phosphorylation of MEF by the cyclin A-CDK2 complex controls its transactivation activity during the cell cycle, establishing a novel link between the ETS family of proteins and the cell cycle machinery.

Cyclin-dependent kinases (CDKs) play a critical role in the commitment of a cell to proliferate; commitment occurs during the G1 to S transition, at a time called the restriction point. CDKs are activated by their association with regulatory subunits known as cyclins. The transition from G1 to S requires the temporal activation of cyclin D-CDK4 or cyclin D-CDK6, cyclin E-CDK2, and cyclin A-CDK2, while cyclin B-CDK1 (CDC2) is essential for the G2/M transition (3). Cyclin D- and cyclin E-dependent kinases regulate the activity of the transcription factor E2F, which controls the expression of a variety of genes necessary for DNA synthesis (4, 5). Cyclin-CDK complexes phosphorylate the retinoblastoma gene product (Rb), releasing E2F from its sequestration by Rb and allowing E2F to transactivate genes essential for S phase, such as DNA polymerase-α, thymidine kinase, and cyclins E and A. Both cyclin E-CDK2 and cyclin A-CDK2 can form quaternary complexes with the Rb-related protein p107 and with E2F during S phase; the exact function of these complexes remains obscure (6, 7).

The ETS family of transcription factors has been shown to play key roles in the growth and differentiation of both hematopoietic and nonhematopoietic cells. Disruption of PU.1 or Ets-1 by homologous recombination can block the development of granulocyte and monocyte lineages or reduce the number and proliferative capacity of mature T cells (8–12). Overexpression of Fli-1 or PU.1 can generate murine erythroleukemias (13, 14), whereas morphogenic abnormalities that resemble Down’s syndrome are seen in transgenic mice that overexpress Ets-2 (15). ETS genes are involved in numerous, specific chromosomal translocations found in human leukemias and sarcomas (e.g. TEL-AML1, TEL-PDGFRB, EWS-FLI1, EWS-ERG, and EWS-ETV1 (16–20).

We recently cloned an ETS-related factor called MEF (myeloid elf-1-like factor), which is expressed in a variety of hematopoietic and nonhematopoietic cells (21). MEF binds to DNA in a sequence-specific fashion and potently transactivates the granulocyte-macrophage colony-stimulating factor and IL-3 promoters. We have demonstrated that MEF physically and functionally interacts with members of the AML1/RUNX1 family of hematopoietic regulatory proteins and showed that its activity is repressed by the t(8;21) AML-associated fusion protein AML1-ETO (22). Very recent studies of MEF homozygous null mice (−/−), generated by homologous recombination, demonstrated that the transcriptional activity of MEF is largely restricted to the G1 phase of the cell cycle.

The abbreviations used are: CDK, cyclin-dependent kinase; Rb, retinoblastoma; IL, interleukin; Tk, thymidine kinase; CAT, chloramphenicol acetyltransferase; GFP, green fluorescent protein; CMV, cytomegalovirus; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay; HPLC, high performance liquid chromatography; FACS, fluorescence-activated cell sorting; aa, amino acids.
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modified Dulbecco’s medium with 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics and were trypsinized and re-plated the day prior to transfection. Ten µg of the empty MEF expression plasmid pEGFP-C3 (CLONTECH) was co-transfected with pCMV5 expression vector. Two micrograms of the reporter plasmid pEGFP-C3 (CLONTECH) which is regulated by the MEF unresponsive CMV promoter, rather than 10 µg, were used for the experiments shown in Fig. 1. Because of the stronger fluorescent signal, 20–24 h after transfection, the cells were harvested for CAT assay (performed as described previously) and transfection efficiency was determined by actin expression. To assess for green fluorescence-positive cells, COS cells transfected with a GFP or EGFP reporter plasmid were Trypsinized 24 h after electroporation, washed with phosphate-buffered saline (PBS), and resuspended in phenol red-free Dulbecco’s modified Eagle’s medium (high glucose) with 5% FBS. The intensity of green fluorescence in these cells was analyzed, and cells were sorted into various fractions using a FACScan (Becton Dickinson) fluorescence-activated cell sorter. The cell cycle profile was analyzed using a FACStar (Becton Dickinson) flow cytometer, after nuclear preparation and staining with ethidium bromide (34).

Preparation of Anti-MEF Antiserum—Anti-MEF antiserum was generated by immunizing rabbits with the C-terminal portion of bacterially expressed MEF protein (amino acids 420–463) generated by the pET-30b(+) expression plasmid (Novagen) and purified using nickel-agarose beads. The antiserum was further purified using Affi-Gel 10 (Bio-Rad) columns coupled to the same antigen.

Expression of Proteins in Sf9 Insect Cells Using Recombinant Baculoviruses—The cDNA encoding the MEF protein was subcloned into the pBlueBac-HisB plasmid (Invitrogen), and recombinant baculovirus was generated that expresses MEF protein with a histidine tag. Recombinant baculoviruses that express cyclin A and CDK2 were kindly provided by D. O. Morgan (35), and those that express cyclin D and CDK4 were kindly provided by C. J. Sherr (36).

Labeling of Cells with [35S]Methionine and Immunoprecipitations—The Sf9 insect cells were plated in six-well, tissue culture plates at 1.5 × 10⁶ cells per well and infected with recombinant baculoviruses. After 48 h of infection, the cells were metabolically labeled with 200 µCi of [35S]methionine (ICN) for 3 h in Grace’s medium (GIBCO-BRL) supplemented with 10% dialyzed fetal bovine serum (FBS) but without methionine. Cells were washed with PBS once, lysed with Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 8, 1 mM EDTA) in the presence of protease inhibitors (10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride), and then clarified by centrifugation. After incubation with specific antibodies against MEF, cyclin A (Santa Cruz), or CDK2 (Santa Cruz) for 1 h, protein A-Sepharose beads (Amersham Pharmacia Bio- tech) were added to the extracts and mixed for 45 min at 4°C. The immune complexes were washed four times with Nonidet P-40 lysis buffer, once with high salt buffer (1 mM NaCl, 20 mM Tris-HCl, pH 8), and once with low salt buffer (10 mM MgCl₂, 50 mM HEPES-KOH, pH 7.5); they were released by adding SDS sample buffer.

To detect the protein complexes in vivo, 24 h after electroporation, COS cells transfected with the expression plasmids for MEF, cyclin A, and p21 were labeled with 200 µCi of [35S]methionine for 4 h in Sf9 cell’s modified Dulbecco’s medium minus methionine with 10% dialyzed FBS. The immunoprecipitations were performed as described above. After four washes with Nonidet P-40 lysis buffer, the immune complexes were released from protein A-Sepharose beads by adding 100 µl of denaturating buffer (50 mM NaCl, 10 mM Tris, pH 8, 5% SDS, 1 mM DTT) and incubated at 95°C for 5 min. Protein complexes were sequentially immunoprecipitated, using specific antibodies, in IP-2 buffer (150 mM NaCl, 20 mM Tris, pH 8, 1% Nonidet-P-40, 0.25 mM phenylmethylsulfonyl fluoride), were recovered with protein A-Sepharose beads and were washed four times with IP-2 buffer. After SDS-PAGE, the gels were dried and exposed to Kodak XAR film at room temperature.

To detect the in vivo interaction between endogenous MEF and the cyclin A-CDK2 complex, Kasumi-1 cells (a human acute myeloid leukemia cell line) were synchronized at the G₁/S phase of the cell cycle by treatment with 2 mM hydroxyurea for 24 h. The cells were then lysed with NETN buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 8, and 1% Nonidet-P-40), and the lysates were subjected to immunoprecipitation using rabbit preimmune serum, anti-MEF, anti-CDK2, and anti-Cyclin A antibodies, respectively. The immunoprecipitates were electrophoretically separated using SDS-12% PAGE, and MEF protein was detected by immunoblotting using an anti-MEF antisera.

In Vitro Kinase Assay—Full-length MEF protein was expressed in Sf9 cells using recombinant baculovirus, then purified and fixed on nickel-agarose beads (Novagen). After 48 h of infection, cells were washed with PBS, then lysed with high salt lysis buffer (500 mM NaCl, 20 mM Tris-HCl, pH 8, 0.5% Nonidet-P) containing proteinase inhibitors (10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride), and clarified by centrifugation. Imidazole was added to the lysate to a final concentration of 40 mM, and the cellular extract was mixed with nickel-agarose beads for 18 h. After washing with 1× binding buffer (500 mM NaCl, 20 mM Tris-HCl, pH 8, 20 mM imidazole) and washing buffer (500 mM NaCl, 20 mM Tris-HCl, pH 8, 60 mM imidazole), beads carrying MEF protein were resuspended in NT buffer (150 mM NaCl, and 20 mM Tris-HCl, pH 8). Two microliters of Sf9 cellular extract containing a combination of cyclin D2 and CDK4, cyclin E and CDK2, or cyclin A and CDK2 were incubated with 0.2 µg of GST-Rb (Santa Cruz) or 20 µl of nickel-agarose beads bearing histidine-tagged MEF protein in a 30-µl kinase reaction...

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mixture (50 mM HEPES-KOH, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 10 mM β-glycerophosphate, 0.1 mM Na₂VO₃, 1 mM NaF, 20 μM lithium-ATP, and [γ-³²P]ATP) for 30 min at 30 °C. After adding 10 μl of 4× SDS sample buffer, samples were boiled for 5 min at 95 °C, then separated by SDS-PAGE, and autoradiography was performed. The relative intensities of each band was determined using a Fuji image analyzer, and the ratio of MEF:Rb phosphorylation for each reaction was calculated. In later experiments, a histidine-tagged mutant MEF protein (MEF-TRI-A) was evaluated for its ability to be phosphorylated by cyclin A-CDK2 in vitro.

Electrophoretic Mobility Shift Assays (EMSAs)—MEF, cyclin A, and CDK2 proteins were expressed in Sf9 cells, in various combinations, using recombinant baculoviruses. After 48 h of infection, cells were washed with PBS, resuspended in buffer S (20 mM HEPES-KOH, pH 7.5, 50 mM KCl, 1 mM DTT) containing proteinase inhibitors and phosphatase inhibitors (mentioned above), then disrupted by repeated freeze-thawing. The extracts were clarified by centrifugation, and the amount of MEF protein in each extract was assayed by Western blotting, using serially diluted samples.

Complementary strands of the APET oligonucleotide were annealed and labeled using polynucleotide kinase (Roche Molecular Biochemicals) and [γ-³²P]ATP. One ng of labeled oligonucleotide was incubated with Si9 cellular lysates containing a constant amount of MEF protein with or without cyclin A and/or CDK2 protein in a 25-μl reaction mixture containing 20 mM HEPES-NaOH, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 2 mM DTT, 10% glycerol, and 2 μg of poly(II-DC) for 30 min on ice. Samples were loaded onto a 5% nondenaturing polyacrylamide gel and run in 0.5X TBE buffer (Tris borate-EDTA) at room temperature. Gels were dried, and autoradiography was performed with an intensifying screen at ~70 °C.

Determination of Phosphorylation Sites Using Mass Spectrometry—The Sf9 cells infected with recombinant baculoviruses that overexpress MEF protein, and the cyclin A-CDK2 complex, were lysed with K-lysis buffer (150 mM NaCl, 50 mM HEPES-KOH, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.5% Nonidet-P 40, 10% glycerol) containing protease inhibitors and phosphatase inhibitors mentioned above. MEF and cyclin A-CDK2 complex were immunoprecipitated and recovered from the lysates using specific antibodies. The immunoaffinity system was then incubated with and without cyclin A and/or CDK2 protein in a 50-μl kinase reaction mixture at 30 °C. After a 1-h incubation, followed by brief centrifugation, the protein A-Sepharose beads were re-suspended in 45 μl of 2× SDS sample buffer, then heated at 95 °C for 5 min. Samples were separated by SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane.

The bands of interest were excised from the nitrocellulose and processed for internal amino acid sequence analysis as described (37), with modifications (38). Briefly, in situ proteolytic cleavage was done using 0.2 μg of trypsin (modified sequencing grade; Promega) in 25 μl of 100 mM NH₄HCO₃ (supplemented with 1% Tween-20; 3-16) for 2 h at 37 °C. The resulting peptide mixture was reduced and alkylated with 0.1% β-mercaptoethanol (Bio-Rad) and 0.3% 4-vinyl pyridine (Aldrich), respectively, and fractionated by reversed phase HPLC (39). An enzyme blank was done on an equally sized strip of nitrocellulose.

Purified, ³²P-labeled peptides (selected by scintillation counting) were analyzed by a combination of automated Edman degradation and matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry; details about this combined approach can be found elsewhere (39, 40). Chemical sequencing (on 90% of the sample) was done using a model 477A instrument from Applied Biosystems. Stepwise liberated phenylthiohydantoin-derivative acids were identified using an "on-line" 120A HPLC system (Applied Biosystems) equipped with a PTH C18 (2.1 × 220 mm; 5-micron particle size) column (Applied Biosystems). Instruments and procedures were optimized for fentomole level phenylthiohydantoin-derivative analysis as described (39, 40). Theoretical monoisotopic masses of predicted tryptic MEF peptides were calculated using ProComp version 1.2 software (obtained from Dr. P. C. Andrews, University of Michigan, Ann Arbor, MI); to account for possible phosphorylation, 79,366 Da (or multiples thereof) was added to the predicted masses for comparison with experimental values.

RESULTS

The Transcriptional Activity of MEF Varies during the Cell Cycle—Several methods have been used to study changes in the transcriptional activity of proteins during the cell cycle, but these often involve serum deprivation or the use of reversible agents that block the cell cycle. Although these techniques synchronize the growth of cells in culture, they can alter cell physiology in other subtle or not so subtle ways. To overcome this problem, we developed a reporter system based on the detection of GFP (41), whose expression is governed by a MEF-responsive promoter.

The reporter plasmid, called APET-Tk-GFP, contains MEF-responsive enhancer sequences (which we call APET) derived from the human IL-3 5'-flanking region in front of the herpes simplex virus Tk promoter. The sequence-specific binding of MEF to these IL-3 sequences was shown previously (21), and this reporter system accurately measures the transcriptional activity of MEF. The APET-Tk-GFP reporter plasmid has minimal background activity in the absence of MEF, and a mutant APET-Tk-GFP reporter plasmid that lacks the MEF binding site has minimal activity in the presence or absence of MEF (see below). These features enable us to fractionate cell populations by fluorescence-activated cell sorting (FACS) according to the level of GFP expression, which correlates directly with the amount of MEF activity.

Cells were transfected with the APET-Tk-GFP reporter plasmid and an MEF expression plasmid and were separated by FACS sorting into three fractions 24 h later, based on the level of green fluorescence (Fig. 1A). Cell nuclei in each fraction were then stained with ethidium bromide, to analyze the cell cycle distribution by flow cytometry. Approximately 13% of the total cells (the sum of the high (H), middle (M), and low (L) fractions) exhibited significant fluorescence, reflecting the transactivating activity of MEF. Cells with the greatest intensity of fluorescence showed a 2.0- or 3.6-fold enrichment in the number of cells in the G1 phase of the cell cycle, compared with the unsorted total cell population (high (H)/total) or the cells with low intensity (high (H)/low (L)), respectively (Fig. 1A). Thus, the transactivating activity of MEF, as defined by the intensity of fluorescence, clearly correlated with the proportion of cells in G1. As shown in Table I, cells transfected with the APET-Tk-GFP reporter plasmid and an empty expression plasmid did not contain a significant number of cells in the high, medium, or low intensity fractions, and cells transfected with the MEF expression plasmid and the ETS mutant APET-Tk-GFP reporter plasmid (with mutated MEF binding sites) did not display any high intensity fluorescence. The peak activity of MEF during G1 suggests that MEF transactivating activity is regulated in a cell cycle-dependent fashion, being higher in G1 than during the rest of the cell cycle. Another explanation is that overexpression of MEF in COS cells causes the accumulation of cells in G1.

To distinguish between these two possibilities, we transfected COS cells with 2 μg of a GFP reporter plasmid that is driven by the CMV promoter (which is not regulated by MEF) and 10 μg of either the MEF expression plasmid or the empty expression vector (Fig. 1B). DNA content was measured in the total and the high, medium, and low intensity GFP-positive fractions; 52–56% of the transfected cells were GFP-positive, reflecting the transfection efficiency. In this setting, the presence or absence of MEF did not alter the distribution of cells throughout the cell cycle (Fig. 1B). This suggests that overexpression of MEF does not cause accumulation of COS cells in G1. Rather, MEF transcriptional activity appears to be regulated during the cell cycle, causing the significant enrichment of the green fluorescence signal in G1.

Modulation of the Transcriptional Activity of MEF by Cyclin A—To further investigate the link between MEF activity and the G1 phase of the cell cycle, we examined whether the forced expression of cyclin D, E, A, or B affected the transactivating activity of MEF using a CAT reporter plasmid under the control of the same MEF-responsive enhancer element (APET-Tk-CAT). MEF transactivated the APET-Tk-CAT plasmid 8–12-
Phosphorylation of MEF by Cyclin A-CDK2

Figure 1. MEF activity is highest during the G1 phase of the cell cycle. A COS cells were co-transfected with the MEF expression plasmid and the APET-Tk-GFP reporter plasmid, so that GFP expression was regulated by the activating activity of MEF. GFP-positive cells were fractionated (into low (L), medium (M), and high (H) fractions) and sorted by FACS according to the intensity of green fluorescence (upper panel). The middle panel shows the histogram of the DNA content in cells within each fraction, and the table in the lower panel reports the percentage of cells in each phase of the cell cycle. The reporter plasmids used for these experiments are shown at the top of A and B. B, COS cells were transfected with 2 μg of a GFP expression plasmid driven by the MEF-unresponsive CMV promoter and 10 μg of either the MEF expression plasmid or the empty vector. Again, the GFP-positive cells were sorted into low (L), medium (M), and high (H) fractions, then the cell cycle distribution of the total cells and the sorted cells was analyzed. The percentage of cells in each phase of the cell cycle is shown.

TABLE I

<table>
<thead>
<tr>
<th>Expression plasmid</th>
<th>Reporter plasmid</th>
<th>Total</th>
<th>High</th>
<th>Medium</th>
<th>Low</th>
</tr>
</thead>
</table>
| Empty vector | APET-Tk-GFP | 1.3
| MEF | APET-Tk-GFP | 4.1
| MEF | APET-Tk-GFP | 5.2
| MEF | APET-Tk-GFP | 6.3
| MEF | APET-Tk-GFP | 7.2

*Cell sorting was not done due to the small population of cells in each fraction.

Phosphorylation of MEF by the cyclin A-CDK2 complex. The transcriptional regulatory activity, DNA binding activity, or multiprotein subunit assembly of several ETS proteins can be controlled, at least in part, by phosphorylation. The ERK, JNK, and p38 members of the mitogen-activated protein kinase (MAPK) families, and casein kinase II, can phosphorylate and regulate these activities of ETS proteins such as ELK-1, SAP-1, or PU.1 (43–46). Thus, it is possible that cyclin A-dependent kinase activity, presumably CDK2, negatively regulates the transcriptional activity of MEF.

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To determine whether CDK can phosphorylate MEF in vitro, we expressed histidine-tagged MEF protein (His-MEF) in Sf9 insect cells using recombinant baculovirus. Following purification, either GST-tagged retinoblastoma protein (GST-Rb) or His-MEF was incubated with extracts from Sf9 cells infected with cyclin D2-CDK4, cyclin E-CDK2, or cyclin A-CDK2 recombinant baculoviruses in the presence of [γ-32P]ATP (Fig. 3A). Extracts containing cyclin D2-CDK4, cyclin E-CDK2, or cyclin A-CDK2 efficiently phosphorylated GST-Rb, as expected (3). In contrast, MEF was most efficiently phosphorylated by cyclin A-CDK2; cyclin D2-CDK4 had negligible activity, whereas cyclin E-CDK2 demonstrated an intermediate ability to phosphorylate MEF. Control extracts, from uninfected Sf9 cells, did not phosphorylate His-MEF or GST-Rb. The ratio of MEF phosphorylation to Rb phosphorylation by each cyclin-CDK complex demonstrates that MEF is a preferred substrate for the in vitro kinase activity of cyclin A-CDK2.

MEF Physically Interacts with Cyclin A—Both cyclin A and cyclin E can form stable complexes with p107, which is then phosphorylated by the cyclin A-CDK2 or cyclin E-CDK2 complex (4). D-type cyclins specifically associate with Rb (38), demonstrating that CDK substrates can bind with high affinity to the relevant cyclins. This prompted us to examine whether MEF can bind directly to cyclin A, MEF and cyclin A, with or without CDK2, were expressed in Sf9 cells using recombinant baculoviruses. The Sf9 cells were then labeled with [35S]methionine, and cell lysates were immunoprecipitated using anti-cyclin A, anti-CDK2, or anti-MEF antibodies. A 100-kDa protein (MEF) was detected in total cell lysates and immunoprecipitates from cells infected with an MEF-expressing baculovirus using anti-MEF antiserum; this protein co-immunoprecipitates from cells infected with an MEF-expressing baculovirus.
translated MEF, and it is not observed in uninfected Sf9 cell lysates (data not shown). Similarly, expression of cyclin A or CDK2 in Sf9 cells generated a 60- or 33-kDa protein, as detected using the respective, specific antibody (Fig. 3B). Following co-expression of MEF and cyclin A in Sf9 cells, the anti-MEF antibody co-immunoprecipitated cyclin A, and reciprocally, the anti-cyclin A antibody co-immunoprecipitated MEF protein (Fig. 3B, upper panel), demonstrating that these proteins are present in the same complex in Sf9 cells. No interaction was observed between MEF and CDK2 when these proteins were co-expressed in Sf9 cells without expressing cyclin A (Fig. 3B, lower panel).

The association between MEF and cyclin A was also detected in COS cells transfected with expression plasmids for MEF, cyclin A, and p21 proteins (Fig. 4A). COS cells transfected with these expression plasmids were labeled with [35S]methionine, lysed, and then sequential immunoprecipitation was performed using specific antibodies against cyclin A or MEF. Immune complexes recovered using the first antibody and protein A-Sepharose were released in the presence of 0.5% SDS, then a second immunoprecipitation was performed using these supernatants and a second antibody. Cyclin A was immunoprecipitated using the anti-MEF antibody (lane 1), and MEF was immunoprecipitated using an antibody against cyclin A (lane 2), clearly demonstrating the association of MEF with cyclin A in vivo.

To show the in vivo interaction between endogenous MEF and cyclin A or CDK2, we synchronized Kasumi-1 cells at S phase with hydroxyurea, to enrich cells expressing cyclin A. Kasumi-1 cell lysates were then prepared and subjected to immunoprecipitation with pre-immune serum, or anti-MEF, anti-CDK2, or anti-cyclin A antisera. As shown in Fig. 4B, both the anti-CDK2 (lane 3) and the anti-cyclin A antiserum (lane 4) can co-precipitate MEF, whereas pre-immune serum (lane 1) does not. The position of the endogenous MEF is shown in lane 2. This confirms our finding that MEF interacts with the cyclin A-CDK2 complex in vivo and is a physiological substrate of the complex.
duced Phosphorylation—To determine how the transcriptional activity of MEF is reduced by the presence of cyclin A and its associated kinase activity, we performed EMSA using Sf9 total cellular extracts that contain either MEF alone or MEF with CDK2/cyclin A (Fig. 5A). Cell extracts containing MEF protein generate a specific gel-shift band using the APET oligonucleotide (21) (lane 2). Extracts containing MEF and CDK2, without overexpressed cyclin A, generated a gel-shift band of similar intensity as extracts containing MEF alone (lane 3), whereas the DNA binding activity of the extract was significantly reduced when MEF was co-expressed with CDK2 and cyclin A (lane 4), which suggests that phosphorylation of MEF protein by cyclin A-CDK2 decreases its DNA binding activity. A uniform amount of MEF was present in each of the last three lanes (Fig. 5B). Extracts from Sf9 cells expressing cyclin A and CDK2 contained significant cyclin A-associated histone H1 kinase activity, while infected cells expressing MEF, or MEF plus CDK2, had negligible histone H1 kinase activity (data not shown).

Determination of the Sites in MEF Phosphorylated by the Cyclin A-CDK2 Complex—To address the effect of phosphorylation on the regulation of MEF activity, we have begun to define the sites in the MEF protein that are phosphorylated by the cyclin A-CDK2 complex. We first performed an in vitro kinase reaction using purified MEF and purified cyclin A-CDK2 complex (these proteins were expressed in Sf9 cells and purified using specific antibodies against MEF or cyclin A, respectively), in the presence of 32P-labeled ATP. The radiolabeled phosphorylated MEF protein or the unlabeled control MEF protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. These proteins were then digested in situ with trypsin, and the resulting fragments separated by micro-bore reversed phase HPLC. Aliquots of each tryptic peptide peak, collected during HPLC analysis of digested 32P-labeled MEF (“P-MEF”), were counted, and the total counts/min was calculated for the entire fraction volume. Two adjacent

**FIG. 4.** In vivo association of MEF and cyclin A. A, COS cells transfected with the expression plasmids for cyclin A, MEF, and p21 proteins were lysed, and sequential immunoprecipitations were performed. Specific antibodies used in the first (1st), and the second (2nd), immunoprecipitations are shown at the top of each lane. A, anti-cyclin A antibody, M; anti-MEF antibody. Protein size markers (in kilodaltons) are indicated on the left. B, Kasumi-1 cell lysates were first immunoprecipitated using rabbit preimmune serum (lane 1) or anti-MEF (lane 2), anti-CDK2 (lane 3), or anti-cyclin A (lane 4) antisera. The immunoprecipitates (IP) were electrophoretically separated using SDS-10% PAGE and then subjected to immunoblotting (WB) using an anti-MEF antiserum.

**FIG. 5.** EMSAs demonstrate decreased binding of MEF to DNA after phosphorylation. A, MEF protein was expressed in Sf9 insect cells with or without CDK2 and/or cyclin A using recombinant baculoviruses (as indicated). EMSA was performed using cellular extracts prepared from infected Sf9 cells and 32P-labeled APET oligonucleotides. The unbound APET oligonucleotide is indicated at the bottom of the gel. The other arrow indicates the position of the sequence-specific gel shift complex. B, the equivalent loading of MEF into the last three lanes is shown.

**FIG. 6.** Tryptic peptide maps of cyclin A-CDK2-phosphorylated MEF (top) and unphosphorylated MEF (bottom). Gel-purified proteins were digested and the resulting peptides fractionated by reversed phase HPLC. Only the relevant sections of the chromatograms are shown (20–60 min). Labeled peaks were analyzed by matrix-assisted laser-desorption/ionization reflectron-TOF mass spectrometry and chemical sequencing, yielding the following results: P-MEF-37, IQHVGGLQPSAS... (MEF 353–390) and IVGGYTxxA N... (trypsin auto-lytic product); m/z = 1880.916 (MEF 641–657 + Phosphate), 3960.640 (MEF 353–390). P-MEF-39, 1800.931 (MEF 641–657). MEF-19, m/z = 3961.150. MEF-21: m/z = 1800.896. Only peaks P-MEF-37 and -38 were found to contain 32P-associated counts.
peptide peaks, P-MEF-T37 and -T38 (shown in Fig. 6, top panel), contained nearly all of the $^{32}\text{P}$ counts above background: 13,025 cpm and 5,736 cpm, respectively. T38 appears as a small shoulder on the T37 peak, and almost certainly represents chromatographic tailing. These fractions, together with the closely eluting P-MEF-T39, were taken for mass and chemical sequencing analysis; tryptic peptides that eluted in corresponding positions during HPLC of an MEF control digest is contained within the region 641–657; due to technical limits of detection using phenylthiohydantoin-derivative identification, we cannot rule out phosphorylation at other sites in MEF.

Mutation of C-terminal Serine and Threonine Residues Partially Abrogates the $G_1$ Restriction of MEF Activity and Decreases Phosphorylation by Cyclin A—Within amino acids 641–657 of MEF are three serine or threonine residues followed by a proline residue that represent potential sites of phosphorylation in MEF. The asterisks mark the serine residues that have been changed to alanine in the MEF-TRI-A mutant.

The mutant MEF-TRI-A protein was co-expressed in COS cells with the APET-Tk-GFP reporter plasmid. GFP positive cells were collected and fractionated as described above; the cell cycle distribution of the cells in each fraction (high, medium, and low green fluorescence) was analyzed and compared with the results generated by wild type MEF protein (Table II). The cell cycle distribution of the total cell populations did not differ significantly from each other following transfection of either wild type MEF or MEF-TRI-A. Among cells transfected with wild type MEF, the “high” fluorescent intensity fraction again showed an enrichment of cells in $G_1$ phase, compared with the total population (1.8-fold; 45.3 versus 25.3%). However, the percentage of cells in $G_1$ in the high intensity fraction of cells transfected with MEF-TRI-A was only slightly higher than the total cell population (35.0 versus 28.9%), and the percentage of cells in $S$ phase was similar in the unselected cells and in the high intensity cells containing MEF-TRI-A. The slight decrease in cells in $G_2/M$ (14.3 versus 21%) is of unclear significance, as we have no specific data suggesting regulation of MEF activity during $G_2/M$.

We evaluated the ability of the MEF-TRI-A protein to be phosphorylated in vitro by cyclin A-CDK2 (Fig. 7). Multiple experiments demonstrate that phosphorylation of MEF-TRI-A by cyclin A-CDK2 is markedly reduced compared with the wild type MEF protein (shown in Fig. 7A). Densitometric analysis of these experiments (Fig. 7C) shows that phosphorylation is reduced by ~75%, and Coomassie Blue staining demonstrated that there was roughly an equal amount of MEF-GST fusion protein during the in vitro phosphorylation process.

TABLE II

| Percentage of green fluorescent positive cells and cell cycle distribution of cells in each fraction |
|---|---|---|---|
| Fraction % | Total | High | Total | High |
| $G_1$ | 25.3 | 45.3 | 28.9 | 35.0 |
| $S$ | 53.4 | 42.1 | 49.4 | 50.7 |
| $G_2/M$ | 21.3 | 12.6 | 21.7 | 14.3 |

FIG. 7. Decreased in vitro phosphorylation of MEF-TRI-A by cyclin A-CDK2. A, bacterially expressed GST MEF or GST-MEF-TRI-A proteins were incubated with purified baculovirus-expressed cyclin A-CDK2 proteins. GST-Rb was used as a positive control. B, equal loading of MEF and MEF-TRI-A is shown by Coomassie Blue staining. C, quantification of the phosphorylation of MEF (using densitometry) is shown graphically ($n = 3$ experiments).

FIG. 8. MEF Structure. A, acidic domain; B, ETS domain; C, serine/threonine-rich region; D, proline-rich region. The arrows indicate the threonine-rich region; the proline-rich region. The asterisks mark the serine residues that have been changed to alanine in the MEF-TRI-A mutant.
protein in each lane (Fig. 7B). When the MEF-TRI-A mutant was tested in co-transfection assays with cyclin A, its downregulation by cyclin A was ~40% less than the down-regulation of wild type MEF (data not shown). Taken in aggregate, these data show that the three Ser/Thr residues within aa 641–657 are largely, but not completely, responsible for the regulation of MEF activity by cyclin A.

**DISCUSSION**

Cell cycle-dependent regulation of transcription factor function is critical for the coordination of a variety of cell fates, including differentiation and programmed cell death. Cyclin A is required for the initiation and maintenance of the S phase of the cell cycle (48, 49). Expression of cyclin A and its associated kinase activity are minimal during G1, begin accumulating at the G1/S boundary, and reach a maximum during G2 (50). The suppression of MEF activity by cyclin A-dependent phosphorylation and the cell cycle-dependent regulation of cyclin A suggests that MEF activity decreases during S phase. This hypothesis was confirmed by using a GFP reporter-based cell sorting method that fractionates transfected cells on a single cell basis according to the level of MEF activity (without chemically perturbing them) as we demonstrated that the transactivating activity of MEF is largely restricted to the G1 phase of the cell cycle. Cells fractionated for high MEF activity are enriched in G1, which likely reflects the increasing activity of the cyclin A-CDK2 complex during movement into S phase, which leads to inhibition of MEF transactivating activity.

The regulation of MEF activity by the cyclin A-CDK2 complex is further demonstrated by the physical interaction between cyclin A and MEF, which was observed between the endogenous MEF and cyclin A proteins in a human leukemia cell line. We confirmed that this interaction is direct using Sf9 cells and COS cells. MEF is a preferred substrate for the cyclin A-CDK2 kinase complex in vitro and CDK inhibitors or a dominant negative mutant of CDK2 abrogated cyclin A-dependent suppression of the MEF activity in transfected cells. These observations strongly suggest that cyclin A-CDK2 is a modulator of MEF activity in vivo, by direct phosphorylation. Although we have demonstrated that the cyclin E-CDK2 complex can modestly phosphorylate MEF, we saw no obvious effect of the cyclin E-CDK2 complex on the transcriptional activity of MEF. Several transcription factors, including Rb, can be sequentially phosphorylated by different cyclin-CDK complexes during the cell cycle (51). Therefore, we do not rule out the possibility that MEF can also be phosphorylated by cyclin E-CDK2 (or even other cyclin-CDK complexes) in vivo.

The percentage of GFP-positive cells in each fractionated cell population depends on the number of cells in that phase of the cell cycle, the activity of MEF, and the relative rates of synthesis and decay of the GFP protein. Since we have shown that expression of MEF does not cause a significant accumulation of COS cells in G1, the amount of fluorescence in G1 largely reflects the transcriptional activity of MEF during that phase. The amount of GFP fluorescence in S phase likely reflects some carryover of GFP protein expression from late G1 phase and some residual MEF activity present at early or middle S phase, which would decrease by the gradually increasing cyclin A-CDK2 activity throughout the S phase. Mutations that eliminate the G1 restriction of MEF activity would be expected to increase the amount of GFP expression in S phase, and after defining a 17-amino acid region of MEF (residue 641–657) that is phosphorylated in vitro by cyclin A-CDK22, we demonstrated that phosphorylation of three potential cyclin A-CDK2 phosphorylation sites in the C terminus of MEF did just that. These aa substitutions greatly attenuated the cell cycle-dependent regulation of MEF activity in vivo, as reflected by the shift of GFP protein expression from G1 toward S phase (Table II). These mutations, which block phosphorylation in this region by cyclin A-CDK2 (and potentially other cellular kinases), do not completely eliminate phosphorylation of MEF by cyclin A-CDK2 nor do they completely abrogate the G1 restriction of its activity. Future studies will determine whether phosphorylation of MEF by cyclin A-CDK2 occurs at any of the four additional potential phosphorylation sites found in MEF (Fig. 8).

It is still unclear how the cyclin A-dependent phosphorylation of MEF decreases its DNA binding activity and whether other cell cycle-dependent mechanisms could also control MEF function. These questions await the determination of the crystal structure of MEF protein and further investigation. MEF is most similar to ELF-1 among the ETS family members, sharing homology in its DNA binding domain and N-terminal region (21). However, unlike ELF-1, MEF does not have a consensus LXCXE motif for binding to Rb. This suggests that cell cycle progression from G1 to S regulates the activities of ELF-1 and MEF by distinct mechanisms.

Cyclin-CDK-dependent inactivation of transcriptional modulators like Rb, involvement of a CDK complex (cyclin H-CDK7) in the basic transcriptional apparatus, or direct phosphorylation of transcription factors by the CDKs, as we describe in this report, can regulate transcription during the cell cycle (2). Phosphorylation by cyclin A-CDK2 has been shown to stimulate the activity of p53 (52, 53) and B-myb (54–56), but inhibit the DNA binding activity of E2F (57–59) and of MEF (this report). The G1-restricted activity of MEF may be critical to control the transcription of genes required for cell fate determination during the restriction point, which can be influenced by cytokines and other extracellular factors (1). The importance of ETS family proteins in the differentiation and proliferation of hematopoietic lineages, as demonstrated by gene targeting experiments and our preliminary analysis of MEF homozygous null (−/−) mice, suggests a role for MEF in both hematopoietic stem cell and lymphoid cell biology. Once cells pass the restriction point, the cyclin A-CDK2 activity that accumulates during late G1 through S will turn off gene transcription that is dependent on MEF. Alternatively, as cells commit to withdraw from the cell cycle and initiate a differentiation program, cyclin A expression and its associated kinase activity remain low, allowing MEF to constitutively stimulate the expression of genes involved in growth arrest and/or differentiation. We are using gene chip microarrays to identify direct MEF target genes, but our study also confirms the importance of post-translational changes in regulating protein function.

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**REFERENCES**

12. Tondravi, M. M., McKercher, S. R., Anderson, K., Erdmann, J. M., Quiroz, M.,
5, 908–918
15. Sumarsono, S. H., Wilson, T. J., Tymms, M. J., Venter, D. J., Corrick, C. M.,
379, 534–537
16. Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Meiot, T., Peter, M.,
Nature 359, 162–165
Genes Dev.
18. Sumarsono, S. H., Wilson, T. J., Tymms, M. J., Venter, D. J., Corrick, C. M.,
379, 534–537
19. Jeon, I. S., Davis, J. N., Braun, B. S., Sublett, J. E., Roussel, M. F., Denny,
33. Koff, A., Cross, F., Fisher, A., Schumacher, J., Legueuille, K., Philippe, M., and
35. Koff, A., Cross, F., Fisher, A., Schumacher, J., Legueuille, K., Philippe, M., and
43. Koff, A., Cross, F., Fisher, A., Schumacher, J., Legueuille, K., Philippe, M., and
47. Koff, A., Cross, F., Fisher, A., Schumacher, J., Legueuille, K., Philippe, M., and
51. Koff, A., Cross, F., Fisher, A., Schumacher, J., Legueuille, K., Philippe, M., and
55. Koff, A., Cross, F., Fisher, A., Schumacher, J., Legueuille, K., Philippe, M., and
59. Koff, A., Cross, F., Fisher, A., Schumacher, J., Legueuille, K., Philippe, M., and
63. Koff, A., Cross, F., Fisher, A., Schumacher, J., Legueuille, K., Philippe, M., and
71. Koff, A., Cross, F., Fisher, A., Schumacher, J., Legueuille, K., Philippe, M., and