LEK1, a member of the LEK family of proteins, is ubiquitously expressed in developing murine tissues. Our current studies are aimed at identifying the role of LEK1 during cell growth and differentiation. Little is known about the function of LEK proteins. Recent studies in our laboratory have focused on the characterization of the LEK1 atypical Rb-binding domain that is conserved among all LEK proteins. Our findings suggest that LEK1 potentially functions as a universal regulator of pocket protein activity. Pocket proteins exhibit distinct expression patterns during development and function to regulate cell cycle, apoptosis, and tissue-specific gene expression. We show that LEK1 interacts with all three pocket proteins, p107, p130, and pRb. Additionally, this interaction occurs specifically between the LEK1 Rb-binding motif and the "pocket domain" of Rb proteins responsible for Rb association with other targets. Analyses of the effects of disruption of LEK1 protein expression by morpholino oligomers demonstrate that LEK1 depletion decreases cell proliferation, disrupts cell cycle progression, and induces apoptosis. Given its expression in developing cells, its association with pocket proteins, and its effects on proliferation, cell cycle, and viability of cells, we suggest that LEK1 functions in a similar manner to phosphorylation to disrupt association of Rb proteins with appropriate binding targets. Thus, the LEK1/Rb interaction serves to retain cells in a pre-differentiative, actively proliferative state despite the presence of Rb proteins during development. Our data suggest that LEK1 is unique among LEK family members in that it specifically functions during murine development to regulate the activity of Rb proteins during cell division and proliferation. Furthermore, we discuss the distinct possibility that a yet unidentified splice variant of the closely related human CENP-F, serves a similar function to LEK1 in humans.

Precise coordination of cell cycle events, apoptosis, and tissue-specific gene expression during development is crucial for the normal growth and maturation of embryonic tissue. Although these processes are generally considered mutually exclusive, their coupling and decoupling play critical roles in signaling several key events during embryogenesis. For example, cell cycle withdrawal triggers tissue-specific gene expression and terminal differentiation of skeletal muscle and adipocyte (1). In contrast, during heart development, proliferation and differentiation are coupled until a few days after birth when cardiac myocytes undergo cell cycle withdrawal and terminal differentiation (2). Whereas appropriate differentiation of embryonic tissues is dependent on regulators of these cellular processes, the precise mechanisms utilized to coordinate the execution of these events during development are still unknown.

Recent studies have focused on the role of cell cycle regulators, specifically the retinoblastoma (Rb) family of transcriptional suppressors and their effectors and effectors, in directing coordination of developmental processes (3-4). Whereas these proteins are classically known for their tumor and cell cycle suppressive properties, more recent studies implicate the involvement of Rb family members and their target proteins in the regulation of, not only proliferative, but apoptotic and differentiative events as well during development of embryonic tissues (3). Several studies implicate the direct involvement of Rb proteins and their binding partners in regulation of these developmental processes (3). For example, up-regulation of p21 cyclin inhibitor expression inhibits the release of pRb from E2F and has been shown in muscle, nerves, and other tissues to be involved in apoptotic protection (5). Furthermore, a decrease in expression of pRb in skeletal muscle and liver results in reversal of cell cycle withdrawal (6,7). Numerous studies have demonstrated that Rb proteins function either alone or in conjunction with E2F transcription factors to induce transcriptional suppression of cell-cycle regulatory genes during development. Particularly, pRb has been shown to regulate the activity of MyoD and c/EBP transcription factors in muscle and adipocyte, respectively (3). In addition, pRb and p130 regulate HBP1 transcriptional repression during osteogenesis (8). These data implicate an alternative role for cell cycle regulators, specifically pocket proteins, in directing complex cellular processes during tissue maturation. However, the mechanisms utilized by Rb family members and associated proteins in the regulation of distinct developmental processes during embryogenesis are poorly understood.

During development, individual members of the Rb family of pocket proteins, pRb, p107, and p130, show both spatial and temporal variations in their expression patterns (9). These variations translate into distinctions in their regulation of tissue-specific differentiation. Genetic analyses have revealed that abnormalities in mice deficient for these genes correlate well with their embryonic expression patterns. For example, high levels of expression of pRb detected in the developing liver and central nervous system of wild-type mice during embryogenesis are consistent with increased apoptosis and defects in the differentiation of hematopoietic and nerve cells observed in Rb-null mice (10). However, little is known about how these pocket proteins are able to regulate developmental processes of individual cell lineages. Furthermore, how these proteins, whose roles in the promotion of cell cycle withdrawal and

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demonstrate that LEK1 can interact with all three membranous, proliferative, and apoptotic events during differentiation. Here LEK1 function and its potential role as a regulator of cell cycle, transactivation domain (16).

LEK1 protein completely lacks amino acid sequences demonstrated in CENP-F/Mitosin to be responsible for kinetochore binding (11, 12). Furthermore, analyses of the genomic structure reveal that this domain is encoded on one exon, suggesting that functional differences among these proteins are the result of splice variations and that splice variations producing orthologous proteins through disruption of their association with other regulatory proteins.

Our studies suggest that LEK1 is not a novel E2F-like transcription factor, but rather functions as an inhibitor of pocket protein-mediated activities during development. The data presented here demonstrate that LEK1 binds the critical “pocket” region of Rb that is responsible for its interaction with target regulatory proteins. Our studies suggest that this interaction disrupts binding of regulatory proteins with Rb and may be an essential step in the shut-down that occurs in the coordination of cell division, differentiation, and apoptosis during development. To determine whether LEK1 activity affects cellular processes normally associated with Rb function, we disrupted LEK1 protein expression in cells using morpholino antisense oligomers that have higher specificity and are more stable than traditional antisense oligomers. These studies reveal that LEK1 depletion causes a decrease in proliferation and increased apoptosis. In addition, an arrest in the G1/S phase of the cell cycle was observed in LEK1-depleted cells. Furthermore, a delay in cell cycle progression occurred causing LEK1-deprived cells to fall one cell cycle behind the control cells. Taken together, these data provide strong evidence that LEK1 functions to regulate cellular processes, such as cell cycle progression and apoptosis, by influencing the activity of pocket proteins through disruption of their association with other regulatory proteins.

**EXPERIMENTAL PROCEDURES**

Construction and Expression of GST fusion Proteins—To prepare the LEK1 fusion protein with a GST tag, a plasmid pZ6 containing COOH-terminal pLEK1 cDNA cloned into pGEM-T Easy (Promega) vector was digested with EcoRI. The excised fragment, which included a ~120-kDa coding region and a 3’ non-coding region of the LEK1 gene, was purified from an agarose gel. The EcoRI-EcoRI fragment of the COOH-terminal LEK1 coding sequence was inserted in-frame into the EcoRI site of the linearized expression vector pGEX-2T (Amersham Biosciences) to generate GST-nLEK. A GST fusion protein, GST-nLEK, that contained an additional NH2-terminal LEK1 sequence was also generated by addition of a ~21-kb LEK1 fragment 5’ of the GST-nLEK sequence. This fragment was produced by standard PCR methods using the primers 5’ BamHI-LEK (5’-TAAAGGATCCAGTATAGGCTGAGGAGTCAGCTGGTCAAG-3’) and 3’ HindIII-LEK (5’TCTGCTGTA-GAAAGTCTGCTG-3’) to amplify the sequence from a LEK1 cDNA clone. The generated fragment was then digested with BamHI and HindIII and ligated to BamHI-HindIII-linearized GST-nLEK.

GST-AMH fusion protein (17) that was used as a positive control for DNA binding experiments was kindly provided by the laboratory of Dr. Kathy Gould (Vanderbilt University Medical Center). GST-Rb (amino acids 379–928), GST-Rb (amino acids 379–792), GST-Rb (amino acids 792–928), and GST-p107 (amino acids 250–936) constructs used in the GST pull down assays were a generous gift from Dr. Pradip Raychaudhuri (18).

Expression and purification of GST-LEK1 fusion and other GST fusion proteins were achieved by the methods described for use of the Bulk GST Purification Module (Amersham Biosciences).

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Expression of GST-LEK1 fusion and other GST fusion proteins were achieved by using the methods described for use of the Bulk GST Purification Module (Amersham Biosciences). E. coli (BL21DE3 pLys-S; Amersham Biosciences) were transformed with GST-nLEK fusion recombinant protein and grown overnight. The cultures were diluted 1:10 in 2×YT medium (16 g/liter tryptone, 10 g/liter, 5 g/liter NaCl) containing ampicillin (100 μg/ml) and incubated 3 h at 37 °C with shaking. Isopropyl β-D-thiogalactopyranoside (Amersham Biosciences) was added to a final concentration of 0.1 mM and the cultures were incubated for an additional 2–3 h at 30 °C with shaking. For analysis of bacterial protein expression, aliquots of cell cultures were pelleted, lysed, and run on 10% SDS-PAGE gels. Proteins were visualized by Coomassie Blue staining.

To recover LEK1 fusion proteins using glutathione-Sepharose (Amersham Biosciences), cultures were pelleted by centrifugation at 5000 × g for 5 min at 4 °C and resuspended in 1/10 volume of cold PBS or NETN buffer. The bacteria were then lysed by mild sonication on ice and centrifuged at 10,000 × g for 5 min at 4 °C. Glutathione-Sepharose

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1. L. Pabon-Peña, E. Dees, M. Ashe, K. L. Price, and D. Bader, unpublished data.
2. R. Goodwin, E. Dees, and D. Bader, unpublished data.
3. The abbreviations used are: GST, glutathione S-transferase; PBS, phosphate-buffered saline; CMV, cytomegalovirus; BSA, bovine serum albumin; TBST, Tris-buffered saline with Tween; FACS, fluorescence activated cell sorter, BrdUrd, bromodeoxyuridine.
beads were added to aliquots of bacterial cell lysates and incubated batch-wise for 30 min at room temperature. The glutathione-Sepharose beads were then washed three times with PBS or NETN buffer and aliquots of beads were screened for conjugation with LEK1 fusion protein using Western blot analysis with either α-LEK1 antisera or α-GST antibodies.

Generation of Wild-type pRB Using the Baculoviral Expression System—Wild-type baculoviral pH42.2 expression clone containing the entire coding region of pRB was a generous gift from Dr. Ellen Fanning (Vanderbilt University Medical Center). Transfection was carried out as described previously (19). Purification of pRB was performed by Ni²⁺ column chromatography. Fractions containing pRB were identified by Western blotting.

In Vitro Cell Culture Binding Assay—GST fusion proteins GST, GST-nLEK, GST-nextLEK, and GST-AML were eluted from glutathione-Sepharose beads by adding 1 bed volume of elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0; Pharmacia) and incubating for 10 min at room temperature for three repetitions. All further manipulations were performed on ice or at 4°C. The eluted fusion proteins were dialyzed in PBS overnight. Aliquots containing 500 μg of protein and protease inhibitors were loaded on a column containing a bed volume of ~1 ml of double-stranded DNA-cellulose slurry (Sigma) (0.25 g dry weight swollen with 2 ml of DNA-cellulose buffer; 10 mM Tris, 1 mM EDTA, pH 7.9) equilibrated with PBS. The DNA-cellulose columns were then washed with ~10 bed volumes of elution buffer at the same flow rate to remove any unbound protein. Elutions of bound proteins were carried out by addition of a linear gradient of 100 mM to 1 M NaCl in PBS in 150-μl increments. Samples of the flow through, wash, and gradient fractions were collected for Western blot analysis.

Transfections—The cytomegalovirus (CMV) expression vector p42.2 was used to drive eukaryotic LMK1 expression in cells. It contained a 4.5-kb carboxyl-terminal LMK1 construct that was generated with a 5′ Mfi site ligated to a FLAG epitope (Sigma) fragment with a 5′ Mlu site and a 3′ Mfi site. This FLAG-tagged construct was cleaved at the 5′ Mlu site adjacent to the FLAG epitope and a Sall site at the 3′ end of the LMK1 clone. This fragment was ligated to a mouse embryonic day 18 (E18) LEK1 sequence from mouse embryonic day 18 (E18) (“LEK1”), generating a protein of 1068 amino acids containing the entire coding region of pRB. The cytomegalovirus (CMV) expression vector p42.2 was used to drive eukaryotic LEK1 expression in cells. It contained a 4.5-kb carboxyl-terminal LEK1 construct that was generated with a 5′ Mfi site ligated to a FLAG epitope (Sigma) fragment with a 5′ Mlu site and a 3′ Mfi site. This FLAG-tagged construct was cleaved at the 5′ Mlu site adjacent to the FLAG epitope and a Sall site at the 3′ end of the LEK1 clone. This fragment was ligated to the expression vectors containing the pRB gene and ligated into pCI-neo (Promega) using the polylinker sites MluI and SalI. The LMK1- and LEK1-expressing vectors were transfected into COS-1, 3T3, and C2C12 cells (ATCC) using lipofectamine (Invitrogen) and incubated overnight with 2 μg of DNA per well. The following day, the cells were washed and lysed with cold PBS, boiled in sample buffer, and resolved by SDS-PAGE. The precipitated proteins were analyzed by Western blotting.

Data presented represent treatment of cells with LEK1-ASMor-50 and LEK1-ASMor-100. Sequence complementary to the initiation codon is underlined. The oligomer was prepared according to the manufacturer's recommendations. After 24–32 h of incubation, the transfected cells were recovered and processed at 4°C as follows. Cells were washed in cold PBS, collected from the tissue culture plates, and lysed in 0.1 ml of RIPA buffer (150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 50 mM Tris, pH 8.0) with mild sonication. Whole cell lysates were recovered and aliquots containing 1–2 μg of protein were precleared with Gammabind (Amersham Biosciences) for 30 min at 4°C. Lysates were collected and incubated at 4°C overnight with 3 μg of either α-LEK1 antisera or antibodies against members of the pocket protein family. Antibody complexes were conjugated to Gammabind beads, washed, and boiled in Laemmli sample buffer. Proteins were resolved using SDS-PAGE and analyzed by Western blotting.

Western Blotting—Protein samples were separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride transfer membrane, pore size 0.45 μm (Millipore). Membranes were blocked with blocking solution (2.5% nonfat milk, 1% BSA, 0.01% sodium azide in TBST). After blocking, membranes were incubated with primary antibody at 4°C overnight for 1–2 h at room temperature and washed three times with TBST. The membranes were incubated with secondary antibody in 2% BSA in TBST for 1–2 h at room temperature and washed three times with TBST. The membranes were developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Roche Diagnostics). Antibodies against pocket proteins (Rb, p107, and p130) were obtained from Santa Cruz. Anti-GST antibodies were acquired from Amersham Biosciences.

Cell Culture—COS-1, 3T3, and C2C12 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with fetal bovine serum (Invitrogen) at a concentration of 10, 10, and 20%, respectively, 100 μg/ml penicillin/streptomycin, and 1-glutamine. N-2-Hepes supplemented from Dr. Geraldine Miller, Vanderbilt University Medical Center) were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen), 100 μg/ml penicillin/streptomycin, and 2 mM l-glutamine, and 10 mM HEPES. All cell lines were maintained in a humidified atmosphere of 95% air, 5% CO₂ at 37°C.

Immunostaining—Morpholino-treated 3T3 cells were plated in 2-well chamber slides at a density of 2 × 10⁵ cells/well and grown in media containing 10% serum. Slides were gently washed with PBS and incubated with 70% ethanol for 10–30 min for fixation. Cells were washed briefly with PBS, permeabilized in 0.25% Triton X-100 for 10 min, and blocked in 2% bovine serum solution overnight at 4°C. Cells were then fixed in 100% acetone at −20°C for 10 min and washed three times with PBS at room temperature and washed three times with TBST. The membranes were incubated with secondary antibody in 2% BSA in TBST for 1–2 h at room temperature and washed three times with TBST. The membranes were developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Roche Diagnostics). Antibodies against pocket proteins (Rb, p107, and p130) were obtained from Santa Cruz. Anti-GST antibodies were acquired from Amersham Biosciences.

We conducted a search for 5′ LMK1 sequence by Blast analysis of the known Mitosin sequence containing the 5′ start site against the Celera mouse data base. Sequence alignments were used to generate primers for reverse transcriptase-PCR amplification of the 5′ LEK1 sequence from mouse embryonic day 10.5 total RNA. The amplified fragment was then cloned into pGem T-easy (Promega) vector and sequenced. The 5′ LEK1 sequence was used to generate two antisense morpholinol oligomers. Morpholinol antisense oligomers have a 6-member morpholino ring bound to each genetic base, rendering the oligomer susceptible to degradation and controlling the levels of specificity than with traditional antisense techniques. The antisense morpholinol oligomers of LEK1 were ordered from Gene Tools, LLC (Corvallis, OR). The sequences used to generate the two antisense oligomers were: 5′-CCATTCTCTCAGGTC-TCAGCCTACATC-3′ for LEK1-ASMor and 5′-AGCTCTCAGACAGAT-CTGGCTTCCG-3′ for LEK1-ASMor-50. Sequence complementary to the target sequence on the oligomer was designed using the Gene Tools protocol and administered to cultured cells using the suggested EPEI delivery system. Control oligomers were provided by Gene Tools. The standard control oligomer provided had the sequence 5′-CCTCTCTCAGGTC-TAGACACATC-3′. This standard control oligomer consisted of an inert sequence with no biological target and no detectable biological activity. Fluorescein-labeled oligomers were used to confirm efficient delivery via fluorescence microscopy. Both morpholinol oligomers were able to produce almost identical phenotypic changes, although LEK1-ASMor-50 was more efficient at inhibiting LEK1 expression. Data presented represent treatment of cells with LEK1-ASMor-50.
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RESULTS

LEK1 Associates with Members of the Rb Family in Vitro—The predicted domain structure for the COOH-terminal peptide product of LEK1 (LEK1) is shown in Fig. 1. Sequence analysis of LEK proteins demonstrates that they all contain a highly conserved E2F1-like Rb-binding domain. More striking is the conservation of specific residues known to participate in E2F/Rb interaction (20). Because LEK1 is primarily expressed in the nuclei of cells (13) and contains a highly conserved E2F1-like Rb-binding domain, we wanted to determine whether LEK1 also binds Rb proteins.

For Rb proteins to bind target proteins, they must be in their hypophosphorylated, active state. Once Rb proteins are phosphorylated, they become inactive and are unable to associate with their target proteins (3). To determine whether LEK1 associates with the hypophosphorylated, or active form of Rb, we performed GST pull-down assays using a GST-conjugated LEK1 construct (GST-2.6) similar to the COOH-terminal peptide product of LEK1 (Fig. 1) that includes the E2F-like Rb-binding domain. The LEK1 construct was co-incubated with hypophosphorylated full-length pRb (19) at increasing concentrations, and the LEK1 protein was precipitated using glutathione-Sepharose beads. Protein complexes were resolved and visualized by Western blot. Fig. 2 demonstrates that COOH-terminal LEK1 forms complexes with hypophosphorylated full-length pRb, as is observed by the presence of hypophosphorylated pRb in the LEK1 precipitate at increasing concentrations. pRb did not interact with GST alone.

To assess whether LEK1 associates with other members of the Rb family, as well as pRb, and whether this association can...
LEK1 Regulates the Activity of Rb Family Members

Fig. 3. LEK1 associates with all three members of the Rb family, pRb, p107, and p130 in vitro. FLAG-tagged LEK1 CMV-expression plasmid p42.2 and CMV-Rb, CMV-p107, or CMV-p130 were transfected into COS-1 cells. A, cell lysates were immunoprecipitated (IP) with antibodies against pRb (lane 1), p107 (lane 2), and p130 (lane 3) and Western blots (WB) were used to detect LEK1 using α-LEK1 antibody. IgG, immunoglobulin G, was used as a negative control. B, conversely, protein complexes were immunoprecipitated using α-LEK1 antibody. Western blot analysis with antibodies against pRb (lane 1), p107 (lane 2), and p130 (lane 3), as indicated, was performed to detect the presence of these proteins in complexes.

occur in a cellular environment, COS-1 cells were transiently transfected with a LEK1 CMV expression plasmid that includes the 130-kDa COOH-terminal portion of full-length LEK1 (p42.2) and expression clones containing pRb, p107, or p130. Whole cell lysates were collected and protein complexes were immunoprecipitated using antibodies specific for each pocket protein. Constituents of these immune complexes were assayed by Western blot (Fig. 3). Our results show that all three members of the Rb family are able to form complexes with LEK1 (Fig. 3A). Experiments using α-LEK1 antisera to perform immunoprecipitations yielded the same results, demonstrating that all three Rb proteins coprecipitate with LEK1 (Fig. 3B). Furthermore, these complexes are able to form within a cellular environment with intact cell cycle machinery. These studies demonstrate that LEK1 associates with not just one, but all three members of the Rb family, suggesting that LEK1 has the potential to affect the activity of all members of the Rb family.

LEK1 Does Not Interact with DNA Directly—LEK1 shares several domain structures with E2Fs that are crucial for their transcriptional activity. These include an E2F-like Rb-binding site, a Myc-type HLH motif responsible for interactions with trans-regulatory proteins, and several leucine zippers with the potential to participate in DNA binding (Fig. 1). To determine whether LEK1 interacts with DNA in a similar manner to E2F transcription factors, DNA binding assays were performed. A nonspecific assay was utilized to test whether LEK1 is able to recognize and associate with random DNA sequences. LEK1 GST fusion proteins and control fusion proteins were co-incubated with cellulose beads conjugated to random DNA fragments. Any DNA-conjugated LEK1 protein was recovered in the bead complexes and eluted using increased concentrations of salt. Eluates were collected and assayed for the presence of protein using Western blot (Fig. 4). Two different GST-LEK1 fusion constructs, GST-nLEK and GST-nextLEK (Fig. 4A), were used in these experiments. The first of these constructs, GST-nLEK, contains the 48-kDa carboxyl-terminal-most portion of the LEK1 sequence that includes the Rb-binding domain and two leucine zippers. The GST-nextLEK construct extends another 78 kDa 5' to GST-nLEK and contains three additional NH2-terminal leucine zippers and the spectrin repeat. The results of the DNA-binding assays show that the smaller GST-nLEK construct does not interact with DNA (Fig. 4B, row a, lanes 1–9). Furthermore, extension of the LEK1 clone to include three additional NH2-terminal leucine zippers is not sufficient for association with DNA (Fig. 4B, row b, lanes 1–9). Therefore, putative DNA-binding peptide sequences of LEK1 do not recognize and associate with DNA sequences alone in the absence of other factors. These results suggest that although LEK1 is nuclear and shares some homology to the E2F family, it cannot recognize and bind to DNA sequences directly.

LEK1 Associates with the Regulatory “Pocket Domain” of Rb Proteins—Rb interaction with regulatory proteins, as well as viral oncoproteins, occurs through the critical COOH-terminal pocket domain. This region contains three subdomains, A (amino acids 379–572), B (amino acids 646–772), and C (772–870), as well as a spacer (amino acids 572–646), that recognize
and associate with specific transcriptional regulators. Several protein binding activities have been identified within this region: the “A/B pocket” (amino acids 379–772) is known to interact with viral oncoproteins E1A, E7, and T-antigen; the “long pocket” (amino acids 379–870), that includes portions of the A, B, and C subdomains, recognizes E2Fs; and the “C subdomain” associates with c-Abl tyrosine kinase (21). Regulators of Rb activity function by targeting the pocket region of Rb and disrupting its associative potential (22). To test whether LEK1 association with Rb proteins occurs via the critical pocket domain, we performed affinity assays utilizing a series of GST-Rb fusion proteins containing varying portions of the pocket domain. Their subdomains and spacer regions display distinct and potentially significant differences in their association with Rb.

We then determined whether LEK1 associates with the pocket region of other Rb family members. p107 and p130 have a very similar pocket domain structure that is distinct from the pRb pocket domain. Their subdomains and spacer regions share a higher degree of homology (over 70%) to each other than they do with pocket subdomains of pRb (only 50% homology) (24). To determine whether LEK1 also associates with the pocket region of divergent members of the Rb family, affinity assays using a GST-p107 fusion protein, GST-p107-(250–936), that includes the entire pocket region (pockets A, B, and C) were performed. Our results demonstrate that LEK1 is not only able to interact with the pRb pocket region, but it readily recognizes and associates with the divergent p107/p130-like pocket region as well (Fig. 5B). The fact that LEK1 associates with all three subdomains of the long pocket, and it binds to all three Rb proteins suggests that its interaction with Rb proteins has the potential to regulate binding of the full spectrum of Rb cofactors to the pocket region.

Rb Pocket Proteins Bind to the E2F-like Rb-binding Domain of LEK1—To determine which region of LEK1 is responsible for interaction with the pocket region of Rb proteins, we performed GST pull-down experiments with various GST-Rb fusion proteins to coprecipitate either the p42.2 LEK1 protein construct (42.2 LEK) or a LEK1 construct that contains a carboxy-
LEK1 associates with all three members of the Rb family (Fig. 5B). Deletion of the E2F-like Rb-binding domain of LEK1 completely disrupts the association between LEK1 and the Rb fusion proteins, including association with the p107 fusion protein. These experiments show that the atypical E2F-like Rb-binding domain of LEK1 is essential for interaction with the pocket region of Rb family members.

**Endogenous Association of LEK1 with Pocket Proteins**—Because LEK1 interacts with the pocket region of Rb proteins in vitro, it is possible that LEK1 influences binding of regulatory proteins to the pocket region of Rb family members. To determine whether LEK1 is able to influence pocket protein function in vivo, we needed to decipher first whether endogenous LEK1/Rb protein complexes exist in living cells. To show whether endogenous LEK1/pocket protein interactions occur, we performed a series of immunoprecipitations from NSO cell extracts using antisera against LEK1. We have previously observed that NSO cells express LEK1. The recovered complexes were analyzed for the presence of pocket proteins by Western blot using pRb, p107, and p130 antibodies. As shown in Fig. 6A, lanes 2, 4, and 6, LEK1 forms complexes with all three members of the Rb family in vivo. When the reverse experiments were performed using antibodies against pocket proteins (anti-Rb, anti-p107, and anti-p130) to immunoprecipitate endogenous complexes, LEK1 protein was detected in a complex with each of the three pocket proteins, confirming that LEK1 associates with all three members of the Rb family (Fig. 6B, lanes 1–4). In contrast, anti-IgG antibodies alone were not able to immunoprecipitate LEK1, pRb, p107 or p130 (Fig. 6A, lanes 1, 3, and 5; B, lane 5).

**Depletion of LEK1 Disrupts Cell Division**—Our studies demonstrate that LEK1 associates with the pocket region of Rb family members that is crucial for interaction with other regulatory proteins. These findings support a possible role for LEK1 in the regulation of pocket protein-mediated regulation of cellular processes such as proliferation, cell cycle, and apoptosis, during growth and differentiation. To determine whether LEK1 regulates cell proliferation, we utilized LEK1-specific morpholino antisense oligomers to disrupt translation of LEK1 mRNA (Fig. 7A). To test for adequate depletion of LEK1 protein by the LEK1 morpholinos, immunostaining using α-LEK1 antibody was performed on 3T3 cells that had been previously treated with morpholino oligomers. Results demonstrate that treatment with LEK1-ASMor-50 morpholino oligomer almost completely abolished LEK1 protein expression when compared with standard control morpholino treatment (Fig. 7B). LEK1-ASMorp morpholino oligomer was not as efficient at depletion of LEK1 protein expression, only suppressing expression by ~50% compared with standard control morpholinos (data not shown). However, both LEK1 morpholino oligomers produced nearly identical phenotypic changes when utilized for these experiments (data not shown), demonstrating that even an incomplete knockdown of LEK1 protein expression is sufficient to disrupt LEK1 function. To determine the effects of LEK1 treatment on proliferation, both 3T3 fibroblasts and C2C12 myoblasts were incubated in the presence of either LEK1 morpholino or a standard control morpholino oligomer. The growth patterns of control and experimental cells were assessed by proliferation assays and FACS analysis. Cell counts were performed to determine differences in proliferation. The obtained data reveal that cells incubated with LEK1 morpholino oligomer display a significant reduction in cell number compared with cells incubated with control morpholino.

**DELETION OF LEK1 INCREASES THE NUMBER OF CELLS IN G1 PHASE**—In order to directly determine whether LEK1 depletion affects cell cycle progression, we performed cell cycle analysis on 3T3 fibroblasts that had been previously treated with LEK1 morpholino or a standard control morpholino (Fig. 7C). These results indicate that depletion of LEK1 protein results in a reduced cell number in both non-differentiating 3T3 fibroblasts and C2C12 skeletal myoblast cells with the potential to differentiate (13). Because we observed a decrease in cell number in LEK1 morpholino-treated cells, we analyzed the effect of LEK1 morpholino treatment on the progression of the cell cycle by FACS analysis. Examination of DNA content of both LEK1 and standard control morpholino oligomer cell populations indicate that 3T3 cells show an accumulation of cell populations in the G1 phase of the cell cycle after treatment with the LEK1 morpholino oligomer, consistent with a G1/S arrest (Fig. 8A). As shown, cells treated with LEK1 morpholino show an abnormal cell cycle distribution with an increase in the proportion of cells in G1, with either a visible (separate from S) or indistinct (included in S) G1/S population of ~10% above the control cells. This accumulation of cells in G1 also correlates with a decrease in the population of cells in S phase in the LEK1 morpholino-treated samples. These findings suggest that LEK1 protein depletion results in cell cycle arrest in the G1/S phase, implying that LEK1 depletion inhibits normal cell cycle progression. To confirm whether LEK1 depletion is decreasing the number of cells entering S phase in a manner consistent with G1/S cell cycle arrest, anti-phospho-histone H3 antibody, a marker of mitotically active cells, was utilized to immunostain morpholino-treated cell populations. Fig. 8B demonstrates that a decrease in the number of cells expressing

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4 K. L. Price and D. Bader, unpublished results.
phospho-histone H3 protein is observed for LEK1 morpholinotreated cells compared with the standard control. Analysis of percentages of phospho-histone H3-positive cells reveal that where ~20% of cells were mitotically active in standard control treated cells, only 6% of cells were mitotically active in the LEK1 morpholino-treated cell samples (Fig. 8C), suggesting a reduction in mitotic cells in the LEK1-depleted cultures that is consistent with a G1/S cell cycle arrest.

Depletion of LEK1 Inhibits G2/M Phase Entry and Increases Apoptosis—To analyze the long-term effect of LEK1 depletion on cell cycle progression, morpholino-treated 3T3 cells were synchronized by serum starvation, released, and collected at 24-h time intervals for analysis of DNA content over 4 days. LEK1 morpholino-treated cells show an initial jump in the relative percentage of cells in G2/M (Fig. 9, A and B) compared with standard control cells during the first day after release from serum starvation. Approximately 12% of LEK1 morpholino-treated cells were in G2/M phase, compared with ~7% of the standard control cells, whereas relative percentages for the remaining cell cycle phases were consistent among the two populations. However, at later time intervals, a drastic reduction in G2/M cells is observed for the LEK1 morpholino-treated cells, reaching levels of at or nearly 0%, whereas the percentages for the other phases of the cell cycle remain consistent with those observed for controls. Therefore, the absence of LEK1 protein is preventing cells from reaching the G2 phase of the cell cycle, which is consistent with a cell cycle arrest occurring at earlier stages of cell division. By the third day, LEK1 morpholino-treated cells show an abundant percentage, over 50%, of cells undergoing apoptosis, which is not observed in standard control populations. By the fourth day, over 60% of cells are actively undergoing apoptosis and cells are still not observed in the G2/M phase of the cell cycle in the LEK1 morpholino cell populations. These data show that LEK1 depletion disrupts progression of cells into later stages of the cell cycle and, subsequently induces apoptosis in cells.

LEK1 Depletion Causes Delay in Cell Cycle Progression—To determine how LEK1 depletion disrupts the progression of cells through specific stages of the cell cycle, morpholino-treated cells were synchronized at G0 and analyzed for BrdUrd incorporation and DNA content. Cells were collected at different time stages corresponding each phase of the cell cycle for one complete cell cycle. 3T3 cells begin to enter G1 phase at ~12 h after release of serum starvation and progress into subsequent cell cycle phases (S, G2, and M) approximately every 3 h (Fig. 10A). As expected, standard control cells show maximum BrdUrd incorporation at 12 h, consistent with cells entering S phase. In contrast, the LEK1 morpholino-treated cells do not reach their peak of BrdUrd incorporation until 15 h after release from serum starvation, suggesting that cell cycle progression is delayed by LEK1 deprivation. Analysis of DNA content of morpholino-treated cells reveals that LEK1-depleted cells demonstrate an approximate 3-h retardation, corresponding to a complete cell cycle phase, in peak activity for each phase of the cell cycle compared with standard control cells (Fig. 10B). Furthermore, the delay appears to involve an accumulation of...
cells in earlier stages of the cell cycle, because the LEK1 morpholino-treated cells are already delayed prior to entry into G1. Only 22% of LEK1 morpholino cells are observed in the G1 phase, compared with 42% for standard control cells, at time 0, and do not peak until 9 h later. Furthermore, as shown previously, low percentages of LEK1-depleted cells in the G2/M phase are observed in the initial 15 h after serum starvation, which is consistent with a cell cycle arrest. The cells appear to accumulate at earlier cell cycle phases. At 15 h post-serum starvation, a synchronous release of this accumulated population into the previously underpopulated G2/M phase occurs, causing the drastic and delayed peak observed in the G2 population for LEK1-depleted cells at 15 h post-serum starvation. These results indicate that the cells are able to overcome the cell cycle arrest and progress through subsequent phases of the cell cycle. However, taken with the previous data, the cells are not able to maintain this recovery over subsequent cell divisions, because the G2/M population is observed to disappear over time (Fig. 9, A and B).

**DISCUSSION**

Cellular proliferation and differentiation are distinct yet interrelated processes during embryogenesis and tumorigenesis. Most cells in the body withdraw from the cell cycle at maturity and remain quiescent unless stimulated to re-enter the cell cycle under tumorigenic or pathological conditions (25). Not surprisingly, many pathways that direct proliferation and differentiation during development are important in tumorigenesis (26). Specifically, Rb proteins and their related targets regulate proliferative and differentiative events during both embryogenesis and tumorigenesis. For example, mutant mouse strains with aberrant pocket protein expression exhibit developmental defects, including disregulated proliferation, cell division, and apoptosis, as well as a predisposition to tumors resulting from improper differentiation and ectopic proliferation of certain cell types (8). Therefore, deciphering how Rb proteins exert their activity during cell growth, differentiation, and maturation will aid in understanding the role of pocket proteins in these processes.

**Fig. 8.** LEK1 depletion results in G1 cell cycle arrest in 3T3 cells. Cells were treated with either LEK1-ASMor-50 (LEK) or standard control (Control) morpholino. A, after three rounds of incubation with morpholino oligomers, cells were harvested for determination of cell cycle distribution by FACS analysis. Results of analysis of DNA content were analyzed by MODFIT software using two paradigms: inclusion of G2 (Visible), or exclusion of G2 (Indistinct), from S phase. B, immunostaining of morpholino-treated cells with anti-phospho-histone H3 antibody was performed. Cells were treated with either standard control (SC) or LEK1 (L50) morpholino and immunostained using anti-phospho-histone H3 antibody (α-H3, red) as a marker of mitotic cells. The nuclei of cells are stained with 4,6-diamidino-2-phenylindole (DAPI) (blue) to reveal whole cell populations. C, the percentage of mitotic anti-phospho-histone H3-positive cells for standard control (SC) and LEK1 (L50) morpholino-treated cells were calculated and graphed. Percentages for H3 positive (H3) and H3 negative (~H3) are shown.
proteins during organogenesis and cancer. Because pocket proteins in the adult are primarily associated with regulation of cell cycle and in the embryo are associated with regulation of a plethora of cellular events, including apoptosis, differentiation, and tissue-specific gene expression, it is suggested that separate mechanisms exist to control pocket protein function in developing versus adult tissues. Because pocket protein function is classically linked to cell cycle inhibition and induction of differentiation, the abundant expression of these proteins during developmental stages seems inconsistent and suggests that some mechanism must exist to suppress their activity in regulating these functions in developing tissues. Our studies on LEK1 indicate that we have identified a potential universal suppressor of pocket protein function during embryogenesis.

**LEK1 Is a Potential Universal Regulator of Pocket Protein Activity**—Our studies implicate LEK1 as a potential regulator of pocket proteins during developmental processes in the embryo. Previous immunohistochemical analysis of the LEK1 protein during development revealed that down-regulation of the expression of LEK1 protein in developing cells correlates with their terminal differentiation and withdrawal from the cell cycle. Furthermore, LEK1 is ubiquitously expressed in all actively dividing, non-terminally differentiated cells during development (13). These studies demonstrated the correlation between LEK1 expression and the maintenance of proliferation. Therefore, the temporal and spatial expression of LEK1 is consistent with it having a role in the coupling of mitotic activity and differentiation. Our current studies link LEK1 to the Rb pathway through all three members of the pocket protein family, pRb, p107, and p130. This interaction takes place with hypophosphorylated pRb, which is the active form capable of binding to its partners. Additionally, LEK1 interacts with all subdomains within the critical pocket region, which is responsible for Rb interaction with target proteins, suggesting that the LEK1/Rb interaction has the potential to be biologically significant. This becomes significant when we consider that the in vitro binding assays were conducted in COS-1 cells that contain significant levels of T antigen in their cellular milieu. Previous studies have demonstrated that binding of T antigen to Rb proteins inactivates them by disrupting their association with E2F transcription factors (27). However, several studies, including those by Sellers et al. (28) demonstrate that Rb proteins can still regulate transcription, differentiation, and proliferation without E2F interaction. Therefore, the ability of LEK1 to interact with all three subdomains of the pocket region of Rb proteins despite the presence of T antigen, and do so in a form distinguishable from E2F1, may suggest that it is capable of interfering with alternate regulatory pathways of Rb other than E2F inhibition. Taken together, these data imply that LEK1 activity with respect to Rb proteins has the capability to be universal. Furthermore, LEK1 could affect the binding of pocket proteins to their binding partners as it binds the entire pocket. Because LEK1 is ubiquitously expressed during development, this would allow LEK1 to control Rb function in a diverse set of tissue types. This can be exemplified by the expression patterns of these proteins during heart development. Analysis of mRNA expression patterns of Rb proteins in cardiomyocytes indicates that LEK1 and p107 mRNA expression in cardiac tissue is high in comparison to other members of the Rb family (2, 13). Therefore, LEK1 function during heart development may primarily be to regulate p107 activity that is predominantly linked to regulation of cell differentiation.

We show that LEK1 associates specifically with the pocket domain of the Rb proteins. Studies have demonstrated that cyclin-cdk complexes phosphorylate sites within the A/B bind-

Fig. 9. **LEK1 depletion induces apoptosis in cells.** 3T3 cells were treated with either LEK1 (L50) or standard control (SC) morpholino oligomer. Cells were then grown in 0.5% serum for 48 h to synchronize populations. Cells were grown in 10% serum and collected over the course of 4 days (D 1–4). Collected cells were stained with propidium iodide and assayed for DNA content by FACS. Cell cycle profiles were analyzed using the MODFIT program. A, histograms of both SC- and L50-treated morpholino cells are shown. Cells with 2–4 N DNA content are depicted in red. Cells with <2 N content were counted as apoptotic and are depicted in green. Debris is shown in blue. B, comparison of cell cycle distributions of morpholino-treated cells over the course of 4 days. Data are representative of results of three separate experiments.
LEK1 Depletion Disrupts Proliferation and Cell Cycle Progression—LEK1 depletion decreases proliferation, disrupts cell cycle progression, and increases apoptosis, similar to the effects of up-regulating pocket proteins (3). We therefore hypothesize that LEK1 has the potential to inhibit pocket protein activity by sequestering the pocket domain, thereby preventing Rb interaction with other target proteins. Many proteins that associate with Rb, such as E2Fs, MDM2, and HBP1 are involved in cell cycle progression (3). Therefore, we suggest that, in a similar manner to phosphorylation, LEK1 may prevent the sequestration, and consequent inhibition of cell cycle-promoting factors, such as E2Fs, by blocking their access to the pocket region of Rb family members. Our current hypothesis concerning LEK1 interaction with pocket proteins and its potential function in the regulation of proliferation and differentiation is illustrated in Fig. 11. As LEK1 sequesters Rb proteins during development, it has a titration effect leaving Rb binding partners, such as E2Fs, unbound and activated. Sequestration of Rb proteins by LEK1 would free up-regulatory proteins to induce transcription and/or activation of genes necessary for progression through Rb cell cycle checkpoints. The ability of LEK1 to interfere with the activity of individual members of the Rb pocket proteins and their associative proteins through competitive binding would depend on the spatial and temporal correlations in expression patterns of these proteins during development. We propose that LEK1, in conjunction with moderate to high levels of pocket protein expression, acts to maintain cell division during organogenesis. As previously mentioned, cardiomyocytes express high levels of p107 and LEK1 at coinciding time intervals during development (13). In accordance with our hypothesis, as long as the levels of LEK1 and p107 are high in developing cardiomyocytes, they continue to proliferate and do not terminally differentiate. As the levels of LEK1 and p107 decline at later stages of cardiomyocyte development, the level of pRb increases and remains high until adulthood, with pRb becoming the primary regulator of cell cycle progression and differentiation. Without competition for binding from LEK1, pRb is able to sequester cell cycle-promoting factors and prevent transcription of cell cycle regulatory genes and, possibly, other genes necessary for inhibition of terminal differentiation.

**Fig. 10.** LEK1 depletion retards cell cycle progression. 3T3 cells were treated with either LEK1 (L50) or standard control (SC) morpholino oligomer. Cells were then grown in 0.5% serum for 48 h to synchronize populations. Cells were assayed for BrdUrd incorporation versus propidium iodide staining. A, percent BrdUrd incorporation at different time intervals following synchronization was plotted for standard control (SC, blue) and LEK1 (L50, pink) morpholino-treated cells. BrdUrd positive cells represent cycling cells that are actively synthesizing DNA. Peak BrdUrd incorporation is demonstrated by the arrowhead for SC and arrow for L50 morpholino-treated cells. B, percentages of cells in each phase of the cell cycle, G0/G1, S-phase, and G2/M were calculated from histograms of the DNA content of morpholino-treated cells. Peak percentages are demonstrated using arrows for standard control and arrows for LEK1 morpholino-treated cells. p value <0.008.
Disruption of LEK1 Alters Normal Cell Cycle Progression—Down-regulation of LEK1 expression correlates with general cessation of mitotic activity in various developing tissues (13). If LEK1 interaction with the pocket domain of Rb proteins inhibits association with cell cycle-promoting target proteins, then we would expect that depletion of LEK1 would inhibit proliferation. The results from our cell culture studies using morpholino oligomers to deplete LEK1 protein are consistent with this hypothesis. Our data reveal that depletion of LEK1 results in decreased proliferation and inhibition of cell cycle arresting cells at G1/S. This is consistent with previously reported data in which the introduction of the cdk inhibitor troglitazone to MCF-7 cells was shown to produce a similar (10%) increase in the number of cells in G1, because of a decrease in phosphorylation, and subsequent activation, of pRb protein (25). This type of arrest is frequently seen in systems where the activity of Rb proteins has been enhanced (8). This supports the idea that LEK1 is acting to prevent Rb proteins from inducing cell cycle arrest by sequestering Rb interaction with target proteins and is consistent with the hypothesis that LEK1 is a universal regulator of pocket protein activity, because the arrest occurs at G1, which is the first Rb checkpoint in the cell cycle. If LEK1 is inhibited from sequestering pRb at G1, the activity of pocket proteins involved in later stages of the cell cycle, such as p107 in G2/M, would not be observed. This would also explain the observed retardation in actively cycling cells in LEK1-depleted cultures. Furthermore, during one round of the cell cycle, LEK1-depleted cells accumulate in phases prior to S, which then leads to a delayed entry of the accumulated cell population into G2. This is consistent with the large G2 peak observed in the cell cycle profiles during the first day after synchronization in the apoptosis experiment. During subsequent days, the G2/M peak disappears in these cells. It appears that the G2/M population is already undergoing a drastic decline in LEK1-depleted cells prior to any apoptotic event, suggesting that these phenotypic events may be distinct. These analyses demonstrate that depletion of the LEK1 protein results in a severe disruption in cell cycle progression that, over time, this depletion has detrimental effects on entry of cells into later stages of the cell cycle.

We have further illustrated that LEK1 depletion results in increased apoptosis of cells. As Rb proteins are known to act in conjunction with p53 to prevent cells from undergoing apoptosis (3), these results would seem inconsistent with an inhibitory role for LEK1 in the regulation of Rb proteins. However, analysis of cell cycle profiles of LEK1-depleted cells also demonstrate a severe retardation in cell cycle progression that, after a few rounds of division, could create sufficient stress within the cell so as to signal the cells to undergo apoptosis.

**Implications for LEK1/Pocket Protein Interaction during Development and Cancer**—The significance of Rb proteins in the regulation of cell division and differentiation during both normal and pathological conditions has been the subject of intense study. Many of the downstream targets of pocket proteins have been identified. However, little is known about the factors upstream of Rb proteins that are responsible for regulating their activity during growth and development. Our initial studies on LEK1 suggest that we may have discovered a potential universal inhibitor of pocket protein activity during development. This is significant because few regulators of Rb proteins during development have been identified. Further studies with LEK1 may aid in elucidating the connection between cell division and differentiation, how pocket proteins control specific events during development, such as cell cycle withdrawal and maturation of cells, and what ramifications this link may have in understanding the connection between development and tumorigenesis.

Our data suggest that LEK1 disrupts Rb function during development. Because the role of Rb family members is primarily to inhibit proliferation and promote differentiation, the high levels of expression of these proteins during development seem paradoxical. Few studies have addressed this dilemma. Our studies suggest that we have identified a possible repressor of pocket protein activity during embryogenesis. This implies that the inconsistency in the high level of pocket protein expression in developing tissues and their role in cell cycle withdrawal and differentiation can be explained if an inhibitory mechanism
exists to prevent pocket proteins from functioning normally during organogenesis. An inhibitory cofactor(s), such as LEK1, could interact with Rb family members, hampering their activity and thus allowing developing cells to remain undifferentiated and proliferative. To this date, the relationship between pocket protein function in developing versus mature tissues is not well understood. The identification of a protein that negatively inhibits Rb proteins in such a manner during development would be the first of its kind. This would provide a mechanism for regulation of pocket protein regulation in developing cells and would have significant impact on our current understanding of pocket protein activity during embryogenesis and its relation to the activity of pocket proteins in mature cells.

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