The Cloning and Analysis of LEK1 Identifies Variations in the LEK/Centromere Protein F/Mitosin Gene Family*

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We report the cloning of a novel murine cDNA, LEK1, that is related to human CENP-F and mitosin and more distantly to chicken CMF1. The proteins from these three organisms have significant homology, yet differ in their temporal, spatial, and subcellular localizations. The human proteins bind the kinetochore in mitotic cells, whereas the chicken protein is found only in skeletal and cardiac muscle and is developmentally regulated. Mouse LEK1 is a single copy gene that codes for two developmentally regulated transcripts. The LEK1 protein is expressed early and ubiquitously in mouse development and is generally down-regulated as development proceeds in a manner that correlates to a cessation of mitosis. In adult tissues, the LEK1 protein is detected exclusively in the pronucleus of the oocyte and was not observed in other actively dividing tissues. Subcellular localization revealed that the LEK1 protein in mitotic cells does not bind the kinetochore. From these data, we hypothesize that chicken CMF1, human CENP-F, mitosin, and mouse LEK1 are members of an emerging family of genes that have important and functionally distinct roles in development and cell division.

CMF1 is a recently described gene whose product has been implicated in early chicken heart myogenesis. The possible function of the CMF1 gene product in the differentiation of cardiac muscle was demonstrated by disruption of CMF1 RNA function (1). These experiments revealed that cardiomyocytes infected with CMF1 antisense-containing retrovirus failed to express stage-appropriate markers of differentiation, whereas their uninfected or vector only-infected counterparts expressed stage-appropriate markers of differentiation, whereas their uninfected or vector only-infected counterparts expressed stage-appropriate markers of differentiation, whereas their uninfected or vector only-infected counterparts expressed stage-appropriate markers of differentiation, whereas their uninfected or vector only-infected counterparts expressed stage-appropriate markers of differentiation.

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The abbreviations used are: CENP, centromere protein; Rb, retinoblastoma; DAPI, 4,6-diamidino-2-phenylindole; NLS, nuclear localization signal; DMEM, Dulbecco’s modified eagle medium; dpc, days postcoitum; RT-PCR, reverse transcription-polymerase chain reaction; dn-, dominant-negative; β-Gal, β-galactosidase.

EXPERIMENTAL PROCEDURES

Cloning, Sequencing, and Sequence Analysis of LEK1—The Access RT-PCR system (Promega) was used to amplify cDNAs from 100 ng of...
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9.5-dpc total ICR (Harlan Sprague-Dawley) mouse heart RNA using two sets of degenerate primers (5'-GGCTNCCAGAATGNTTAAA-3' and 5'-CTTTTGTGATNTGCTGCCACC-3', 5'-TTATAYGAWCCAGCAT- TGT-3', and 5'-TTTCTYATTTTCTATAWTYCTGT-3') from two homologous regions between chicken CMF1 and CENP-F/mitosin. These cDNA fragments were then cloned into the T-easy vector (Promega) and sequenced using an ABI Prism Genetic Analyzer (Perkin-Elmer). Inserts from these clones were then used as probes to screen a whole embryo 8.5-dpc mouse cDNA library (courtesy of the laboratory of Dr. B. L. M. Hogan). Overlapping clones were obtained by using standard protocols for cDNA library screening, RT-PCR cloning, 5'-rapid amplification of cDNA ends, and Genewalking (13) and sequenced as stated above.

Northern Blot Analysis—Using standard protocols (13), 10 µg of mouse, human, and chicken genomic DNA were digested with the indicated (Fig. 2) restriction endonuclease overnight at 37°C as per the manufacturer's instructions (Promega). This DNA was processed for Southern blot analysis. The blot was then prehybridized in RapidHyb (Amersham Pharmacia Biotech) for 1 h at 65°C. A probe corresponding to 7017–7222 nucleotides of the LEK1 transcript random prime-labeled with 50 µCi of [32P]dCTP (Amersham Pharmacia Biotech). This probe was then hybridized to the genomic blot overnight at 65°C. The blot was then washed three times in 2X SSC, 0.1% SDS for 1 h/wash at 55°C and exposed overnight using Biomax intensifying screen and Biomax film (Eastman Kodak Co.). This washing and exposure protocol was repeated at 60 and 65°C. All counts were stripped from the blot with boiling 0.05% SDS. It was then reprobed with the corresponding region of the mouse CMF1 transcript in a similar manner as noted above.

In Vivo Immunofluorescence in Mice—Adult and staged embryonic tissues were collected, and the total RNA was isolated using the Trizol reagent as per the manufacturer's instructions (Life Technologies, Inc.). 10 µg of total RNA was electrophoresed on a denaturing 1% agarose, 2.2 M formaldehyde gel and visualized with ethidium bromide as a loading control. The RNA was transferred to a nylon membrane via capillary action overnight and UV-cross-linked. This blot was probed with the same region of the chicken CMF1 transcript in a similar manner as noted above.

Sequence Analysis of LEK1—Analysis of the nucleotide sequences of chicken CMF1, human CENP-F/mitosin, and mouse LEK1 cDNA showed that mouse LEK1 is more closely related to human CENP-F/mitosin than to chicken CMF1. CMF1, CENP-F/mitosin, and LEK1 proteins share a significant amount of homology at the primary, secondary, and tertiary levels. A prominent feature of these proteins is a preponderance of leucine (L), glutamic acid (E), and lysine (K) amino acid residues in these proteins, approximately 40% of the amino acid composition. Thus, we have named the mouse cDNA LEK1 and tentatively refer to these three proteins as the LEK family of genes.

Like CMF1 (1) and CENP-F/mitosin (2, 3), computer analysis (14) predicts that the LEK1 protein is largely composed of α-helices separated by turns except for the proline-rich, globular C terminus. A number of these α-helices are perfect leucine zippers, whereas others have aliphatic heptad repeats. These secondary structures have been shown to be important mediators of both protein-DNA and protein-protein interactions (15–17). The number and general positions of the leucine zippers (Fig. 1, blue oval) are conserved among these proteins. Another shared feature of the three proteins is their predicted tertiary structures. Using the Coiledcoil program (14), the α-helices of the LEK1 protein are predicted to fold into four coiled coils with intervening turns or loops.

RESULTS

Isolation of LEK1 cDNA Clones—Standard low stringency hybridization techniques were initially employed to isolate the murine CMF1 gene (13). A number of libraries (genomic and cDNA) and hybridization conditions were assayed, none of which produced clones. Consequently, an RT-PCR cloning strategy was developed using two homologous regions between the chicken CMF1 and human CENP-F/mitosins to design degenerate primers (see “Experimental Procedures”). These degenerate primers were used to amplify cDNAs from heart RNA of 9.5-dpc mouse embryos. To obtain larger regions of this mouse transcript, the RT-PCR clones were then used as probes to screen a 8.5-dpc whole embryo library (courtesy of Dr. B. L. M. Hogan). The resulting cDNAs were subsequently cloned and sequenced and found to be approximately 75% identical to the human CENP-F/mitosin cDNAs. A combination of RT-PCR, cDNA cloning, genomic walking, and 5'-rapid amplification of cDNA ends techniques were used to isolate overlapping clones such that the primary structure of the LEK1 transcript could be determined. It should be noted that an antiserum (described below) was developed against a sequence from one of the cDNA clones. Subsequently, this antiserum was used to reclone LEK1, demonstrating the specificity of this antibody.

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Mouse LEK1 protein shares a number of other protein motifs with CENP-F/mitosin and CMF1. As would be expected with such large proteins, there are a number of conserved, potential sites for post-translational modifications, such as glycosylation, phosphorylation, myristylation, and amidation. The most conserved region among the human, mouse, and chicken proteins is the C terminus (Fig. 1). In this region, the LEK1 protein contains a predicted helix-loop-helix dimerization domain (Fig. 1, asterisk), a bipartite nuclear localization signal (Fig. 1, yellow stripe and bracketed) (18), an atypical Rb binding site (Fig. 1, black stripe and bracketed) (3), and a P-loop, which is an ATP/GTP binding domain (Fig. 1, bracketed). These motifs are not only well conserved between the three different proteins but are distributed in a collinear fashion (Fig. 1). Though these proteins do share these abundant similarities, it is important to note that comparisons between them do have regions of significant divergence.

**Genomic Analysis of LEK1—** A Southern blot analysis of mice, human, and chicken genomic DNA was conducted to investigate the number of potential LEK1-like genes in the genomes of other representative vertebrates. Mouse, human, and chicken genomic DNAs were probed with the well conserved 3' region of the LEK1 cDNA. At low stringency conditions (see “Experimental Procedures”), numerous bands were observed in all species.3 By gradually increasing the stringency of the washes, the number and intensity of bands decreased until at 65 °C and 2× SSC, 0.1% SDS, only a single band/lane was observed (Fig. 2A). When this same blot was stripped and reprobed with the corresponding region of the chicken CMF1 cDNA, single bands were also observed (Fig. 2B), although their migration differed from those in the mouse-probed Southern blot (compare the arrows in Fig. 2, A and B). Interestingly, bands with similar mobility were observed in a low stringency wash of the mouse-probed Southern.3 This is consistent with the hypothesis that two homologous genes exist in these organisms, a CMF1-like and a LEK1-like gene.

Expression of the LEK1 mRNA during Embryogenesis—To determine both the size and the temporal/spatial distribution of LEK1 transcripts in the murine tissues, Northern analysis experiments were performed. RNA was extracted from staged mouse embryonic and adult tissues. As seen in Fig. 3A, two bands (approximately 10 kilobases) hybridized to the same well conserved sequence used in the Southern analysis. These two transcripts are most likely the result of alternative products from a single gene as only one band was observed in the Southern blot experiments using the same probe and high stringency conditions. The highest levels of LEK1 expression were observed in the early stages of mouse development: 8.5-dpc whole embryo, 9.5-dpc head, and 9.5-dpc caudal regions posterior to the heart (Fig. 3A). It is interesting to note that the relative levels of the LEK1 message appeared to increase during the course of embryonic murine heart and liver development. In other tissues, such as the developing head and brain, the abundance of the LEK1 transcripts decreased with the age of the embryo (Fig. 3A).

Because LEK1-like proteins such as CENP-F and mitosin have been implicated in the mitotic process in vitro, we explored the expression of LEK1 in the developing heart, where...
Thus, LEK1 is developmentally regulated at both the mRNA and protein levels at this critical time in heart development. The low level of LEK1-positive nuclei seen in N7 is maintained in N21 mouse hearts and remains low in adult hearts. A survey of adult organs and tissues was conducted to determine the pattern of LEK1 protein expression in differentiated tissues. Only rare cells were observed to have LEK1-positive nuclei. Interestingly, LEK1 was not observed in skin and intestinal epithelia, where cell renewal via mitosis is common. In fact, in all of the adult tissues examined thus far, LEK1 has only been found in the pronucleus of the oocyte in the mouse ovary (Fig. 6). However, no LEK1-positive pronuclei are observed in mature sperm cells or any other cells in the seminiferous tubules of the testes (Fig. 6).

Subcellular Localization of LEK1 Protein during Mitosis and Skeletal Myogenesis in Vitro—CENP-F and mitosin have been implicated in the mechanical/structural aspects of mitosis (2–7, 21). Our current study found a correlation between the loss of mitotic activity during development and the down-regulation of the LEK1 protein (Fig. 5). However, this correlation was not maintained in differentiated actively dividing cells of the adult mouse (testes in Fig. 6). To investigate the localization of the LEK1 protein in a system where the process of differentiation can be controlled, we employed the mouse skeletal myogenic cell line C2C12. These cells can be maintained as actively dividing cells in mitogen-rich medium or induced to form mitotically inactive, multinucleated myotubes in differentiation medium (22–25). As shown in Fig. 7, nuclei of individual C2C12 cells were stained with anti-LEK1. Over 95% of mononucleated C2C12 cells had LEK1-positive nuclei (compare red fluorescence with DAPI counterstain, Fig. 7, B and C). There is some minor LEK1 staining in the cytoplasm of some cells. In mitotic cells, the LEK1 protein (Fig. 8) is present in all parts of the cell as the nuclear envelope breaks down in prophase (Fig. 8, column I). At metaphase and anaphase (Fig. 8, columns 2 and 3), the LEK1 protein remains fully cytoplasmic but is absent in the area of the condensed chromosomes as determined by counterstaining with DAPI (Fig. 8). At cytokinesis, the LEK1 protein is localized to an area that is slightly greater than the DAPI-staining region (Fig. 8, last column (Myotube)). With the fusion of C2C12 into myotubes, DNA synthesis and mitosis abruptly stops, and the cells enter G2 (25). As shown in Fig. 8, there are LEK1-positive nuclei present in differentiated myotubes (compare LEK1 Ab and DAPI in the myotube), and increased cytoplasmic staining is observed. Later, however, LEK1 is absent in differentiated C2C12 myofibers. Thus, there is no sharp boundary of LEK1 protein expression as these cells are exiting the cell cycle. This pattern of LEK1 protein localization contrasts with previously published reports for both CENP-F and mitosin in mitotic cells (2–4).

Functional Analysis of LEK1 during Differentiation—Data from the previous sections suggest that the subcellular distribution of LEK1 is dynamic during mitosis and differentiation of skeletal myogenic cells. In addition, our data suggest a general relationship between the presence of LEK1 protein and maintenance of mitosis during embryogenesis. LEK1 distribution during mitosis and differentiation is significantly different from that reported for CENP-F, mitosin, and CMF1, suggesting variation in function. Therefore, in an initial effort to determine LEK1 function during cell differentiation, we sought to overexpress a dominant-negative form of LEK1 in the C2C12 myogenic system. Previous studies have demonstrated that a similar dominant-negative lacking 5′ sequences in mitosin leads to phenotypic alterations in cultured fibroblasts (3).

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1. M. E. Dees, R. L. Goodwin, and D. Bader, manuscript in preparation.
C2C12 cells were co-transfected the dnLEK1 construct and a β-Gal-containing plasmid, control vector, and the β-Gal-containing plasmid, or mock-transfected. Cells were maintained in growth medium for 24 h, switched to differentiation medium for 72 h, and processed for β-Gal staining. In transfected cultures, blue-stained cells were scored as either mononucleated cells or differentiated myotubes in each experiment. Cells in differentiated myotubes rose significantly to 0.46 (significance is $p < 0.0001$). The results of these experiments indicate that dnLEK1 accelerated or enhanced the differentiation of C2C12 myoblasts into myotubes in culture.

**DISCUSSION**

In the present study, we have cloned a novel mouse cDNA, LEK1, that codes for transcripts that are related to human CENP-F/mitosin and more distantly to chicken CMF1. The predicted LEK1 protein has significant structural homology to these other proteins in their primary sequence, the type and location of a number of protein motifs, and their predicted overall protein structure. We present data that show that LEK1 is a single copy gene that codes for two developmentally regulated transcripts. These transcripts code for nuclear proteins that are expressed ubiquitously at high levels early in development when cells are most proliferative (26). Alteration of LEK1 function using a dominant-negative form of the protein leads to phenotypic changes in myogenic cells during differentiation. Thus, although LEK1 is structurally related to the chicken and human genes, our data suggest that it is a unique member of this gene family.
also appears to be punctuate staining in the cytoplasm as well. Myotubes were induced to form by maintaining C2C12 cells in low serum conditions for 4 days. Nuclei in these myotubes are LEK1-positive; there are condensed chromosomes. The arrow shows the location of the midbody, an area that stains with anti-CENP-F and anti-mitosin antibodies (2, 3). Myotubes were induced to form by maintaining C2C12 cells in low serum conditions for 4 days. Nuclei in these myotubes are LEK1-positive; there also appears to be punctuate staining in the cytoplasm as well.

The globular C terminus of LEK proteins contains a number of collagen, conserved motifs. A bipartite nuclear localization signal (NLS) (Fig. 1) is a prominent feature in all three proteins (18). The present data showing nuclear localization suggest that this NLS is functional in the LEK1 protein. Just C-terminal to the NLS is an atypical Rb protein binding domain (Fig. 1), that Lee and co-workers (3) have shown to bind Rb. We have determined that the C terminus of CMF1 can bind E-proteins that are helix-loop-helix transcription proteins. Furthermore, LEK1 and CENP-F/mitosin have a computer-predicted helix-loop-helix dimerization domain (Fig. 1, asterisk) (13). Thus, LEK proteins may participate in the transcriptional activities via this C-terminal domain.

Potential Differences among the LEK Proteins—Independent immunological observations have localized the CENP-F and mitosin proteins to paired foci near the centromeres of chromosomes in the late prophase and metaphase stages of mitosis (2, 3). These researchers have gone further to show that the CENP-F and mitosin proteins bind the outer plate of the kinetochore in dividing cells (4, 6). In the current study, we found that the LEK1 protein is localized differently. As seen in Fig. 8, LEK1 staining is observed throughout the entire cell during these stages of mitosis, with the exception of the condensed chromosomes (columns 2 and 3, compare LEK1 Ab and DAPI). Another notable difference between LEK1 and CENP-F/mitosin is the localization of these proteins during cytokinesis. Anti-CENP-F/mitosin antibodies brightly stained areas near the midbody during cytokinesis. Anti-CENP-F/mitosin antibodies do not stain the midbody in actively dividing C2C12 cells. Additionally, CENP-F/mitosin has been shown to be regulated in a cell cycle-coordinated manner, being up-regulated in late G1/S and down-regulated in late telophase (2, 3), whereas LEK1 is present in the nuclei of cells that are essentially at G0 (myotubes in Fig. 8) (25). In addition, not just cells that are actively dividing but the vast majority of early embryonic cells had nuclear staining throughout the entire nucleus (Fig. 4). This in vivo staining indicates that the LEK1 protein is regulated like CENP-F/mitosin is in HeLa cells. Thus, despite the similarities among the primary, secondary, and tertiary structures of CENP-F/mitosin, CMF1, and LEK1, these proteins differ significantly in their subcellular localizations and temporal regulation in mitotic cells.

Evidence of a LEK Family of Genes—As mentioned above, the chicken, human, and mouse proteins have significant ho-

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[^1]: L. M. Pabón-Peña, R. L. Goodwin, and D. Bader, manuscript in review.
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LEK1 is a developmentally regulated nuclear protein that is down-regulated in the heart when mitotic activity of cardiomyocytes ceases (19, 20). However, we do not see any evidence that LEK1 is part of the general mitotic apparatus because it, unlike CENP-F and mitosin, does not appear to bind to the kinetochore, because it is not associated with the condensed chromosomes and appears to be present in all phases of mitosis. Furthermore, we do not see LEK1 in actively dividing adult tissues such as the seminiferous tubules (Fig. 6) or crypts of the intestinal epithelium. The chicken CMF1 protein has not been found to be part of the mitotic apparatus and is tissue-restricted, being found only in the cytoplasm of heart and skeletal muscle cells.

From these data, we postulate that LEK1 function may be involved in developmental mitosis but not in general cell duplication. The human CENP-F and mitosin proteins may also have developmental roles but are deregulated in transformed cells. This interpretation is consistent with these proteins having been found in a variety of human cancers and autoimmune disorders (8–12), in which the normal expression pattern of CENP-F and mitosin may well be perturbed. Chicken CMF1 is a developmentally regulated, tissue-restricted protein that, like other members of this proposed family, has protein motifs that allow it to interact with other proteins. The LEK family of proteins may provide cytoskeletal structural information in the differentiating cell to the nucleus, where they could participate in transcriptional activities and/or the cell cycle machinery. Potential Functions for LEK1—Our initial experiments using dnLEK1 suggest that disruption of LEK1 function leads to phenotypic changes in myogenic differentiation. Previous studies using similar dn forms of mitosin also demonstrate phenotypic changes in mitotic activity in cultured cells (3). Our data show that differentiation of skeletal myoblasts transfected with dnLEK1 is accelerated or enhanced. It is possible that disruption of LEK1 function alters the relationship between cell division and differentiation in skeletal myogenesis. Many groups have shown that decreasing mitotic activity enhances myogenic potential (Ref. 28 and references within). Alternatively, disruption of LEK1 function may directly promote myogenic differentiation. In either case, the present study suggests LEK1 has a potential role in regulating skeletal myogenesis and may even have a broader role in cell differentiation in embryogenesis.

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