Analysis of CMF1 Reveals a Bone Morphogenetic Protein-independent Component of the Cardiomyogenic Pathway*

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Disruption of the CMF1 function in anterior mesoderm inhibits cardiac myogenesis in avian embryos. In the present study, we show that CMF1 is a member of an emerging family of proteins that includes centromeric protein-F, mitosin, and LEK1. These proteins are characterized by their large size (350 kDa), dynamic subcellular distribution, and potential functions in cell division and differentiation. The current data suggest that CMF1 is a unique member of this family by virtue of its restricted protein expression and variant subcellular distribution. Immunochemical analysis demonstrates that CMF1 protein is expressed in cardiogenic cells prior to the activation of cardiac structural gene products. In addition, we show that expression of CMF1 is not dependent on the bone morphogenetic protein (BMP) signaling pathway during development. Still, CMF1 cannot direct cardiogenesis in the absence of such factors as NKX-2.5. Taken with our previous data, this study suggests that CMF1 is a BMP-independent component of the cardiomyogenic pathway.

Cardiac progenitors are some of the first cells to gastrulate in developing chicken embryo (2). These cells are specified to the cardiogenic cell lineage significantly in advance of the formation of a definitive heart tube (3, 4). Studies investigating the expression of contractile proteins indicate that differentiation of cardiac myocytes occurs after approximately 25 h of development and progresses from anterior to posterior within the cardiogenic mesoderm (5–8). In recent years, several genes encoding transcription factors have been implicated in the restriction of anterior mesodermal cells to the cardiogenic cell lineage. For example, the NKX, MEF2, GATA, and HAND gene families clearly play important and interacting roles in the regulation of vertebrate cardiac myogenesis (9, 10). The expression of regulators such as NKX-2.5 is dependent upon an interaction of cardiogenic mesoderm with endoderm that is mediated by BMP1 signaling (11, 12). Although none of these genes regulates cardiogenic commitment and/or differentiation individually, the deletion of any one of them leads to a deviation in the cardiomyogenic differentiation pathway. The specific cellular mechanisms that are regulated by these factors remain unresolved.

Our laboratory has recently identified a novel transcript, CMF1, that is expressed at high levels in early embryonic hearts (1). Our previous studies have shown that retrovirally mediated disruption of CMF1 function inhibits the expression and/or accumulation of sarcomeric myosin heavy chain proteins (MHC) in differentiating cardiac myocytes. These data suggest that CMF1 may play a regulative role in the commitment and/or differentiation of cardiogenic cells.

Preliminary sequence analysis has determined that CMF1 is related to a recently identified group of proteins, CENP-F, mitosin, and LEK1, which are expressed in a wide variety of cell types and have a generally conserved structure and size (13–15). The predicted secondary structure is also conserved among these proteins consisting mainly of a-helices separated by loops with a globular carboxyl terminus. HEla cell-derived CENP-F and mitosin are identical except for a perfect repeat in the 5'-end of CENP-F and a slightly longer 3'-end in mitosin (13, 14).

Immunocytological analyses of CENP-F and mitosin suggest that these proteins are expressed in all dividing cells in vitro and that they have a dynamic subcellular localization (13, 14). During S phase, these proteins become localized to the nucleus. During mitosis they associate with the kinetochore, and subsequently with the spindle apparatus and midbody, and are rapidly degraded after mitosis. This intriguing expression pattern suggested that CENP-F and mitosin could be involved in the regulation of mitosis. Initial functional analyses support this hypothesis, because a dominant-negative construct of mitosin inhibits the G2/M transition of the cell cycle (14).

Although there is an obvious similarity of predicted structure among these proteins, preliminary studies suggest a heterogeneity of function within this family. Although murine LEK1 is more than 80% homologous to human CENP-F and mitosin, it has a vastly different pattern of expression and subcellular localization. LEK1 is ubiquitously expressed in early mouse development, but there is a general cessation of expression throughout the embryonic cell division subsides (15). Although LEK1 is present in all cardiac myocytes early in heart development, its expression sharply declines after 4 days postpartum when mitosis in myogenic cells ceases (15–17). LEK1 is not expressed in adult proliferative cells such as skin and intestinal epithelia. Additionally, the subcellular distribution of LEK1 varies from that of CENP-F and mitosin, as LEK1 is present in the nucleus of interphase cells. During mitosis, it is not detected in the
kinetochore and midbody but is detected in the cytosol. Preliminary functional analysis indicates that LEK1 influences proliferation and skeletal muscle differentiation.

The present study suggests that CMF1 is a member of the CENP-F/mitosin/LEK1 family. Despite the significant similarities among this group of proteins, CMF1 is unique in that its expression is highly restricted and its subcellular localization is vastly different from other family members. In addition, we show that CMF1 RNA is expressed before cardiogenic differentiation. The current data also demonstrate that CMF1 protein is present in cardiogenic cells before the appearance of structural proteins, and its expression precedes that of muscle structural proteins along the anteroposterior axis of the differentiating heart. Interestingly, early CMF1 expression is not dependent upon BMP signaling. Taken with our previous data indicating that CMF1 is an essential regulator of cardiac myogenesis (1), our current data reveal an essential BMP-independent component in the regulation of cardiac myogenesis.

EXPERIMENTAL PROCEDURES

Animals and Tissues—White Leghorn chicken eggs were purchased from Truslow Farms, Charlestown, MD. Eggs were maintained under high humidity in a 37 °C incubator. Embryos were staged according to Hamburger and Hamilton (18), and all tissues were dissected and processed immediately postmortem. For analysis of BMP signaling, stage 4 cardiogenic mesendoderm was isolated using standard techniques (19) and cultured in the presence or absence of the BMP antagonist, noggin (12). Medium conditioned by noggin-producing or non-transfected COS cells (kindly provided by Dr. Tom Schultheiss, Harvard University) was added to standard culture medium (50% v/v). Cultures were maintained for 24 h and processed for RT-PCR analysis.

Cloning and Sequence Analysis of CMF1—During the course of the current study, we discovered that the 5′-most clone of CMF1 contained an inverted cDNA fused to CMF1 sequences. Therefore, we recombined the 5′ sequences of CMF1 using as a probe pCMF1.2.11.b.1 from an embryonic chick heart library (1). Multiple overlapping clones were identified, and both strands of all cDNA clones were sequenced and analyzed using the MacVector and Expasy programs.

Immunohistochemical Analyses—Rabbit antisera were prepared against selected CMF1 peptide sequences (SGHILDSVKELRSSTPSKYN; Biosynthesis). Antibodies were affinity purified with the immunoprecipitation using standard techniques (20) and applied in Western blot and immunofluorescence analyses. For Western blot analysis, tissues were isolated from stage 6, day 11 embryos, sonicated in protein sample buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol, 0.1% bromphenol blue) and immediately loaded on a 6% SDS-polyacrylamide gel after boiling. After electrophoresis, proteins were transferred to an Immobilon membrane (Millipore) using standard protocols. Membranes were blocked in 2.5% dry milk, 1% bovine serum albumin, 1% Triton X-100 for 1 h at room temperature, and incubated with the affinity-purified anti-CMF1 antibody overnight at 4 °C. After extensive washing with 1× TBST, the membrane was incubated with goat anti-rabbit alkaline phosphatase (Sigma) for 1 h and reacted with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals) from 1–5 min.

For immunofluorescence analysis, chicken and quail embryonic tissues were isolated and immediately placed in O.C.T. compound (Sakura) and frozen in isopentane/liquid nitrogen. Frozen tissues were cryosectioned using a Jung CM 3000 cryostat (Leica) in 8–10 micron increments and collected on gelatin coated slides. Slides were fixed with 7% methanol for 10 min and treated with 0.25% Triton X-100 for 10 min. Nonspecific binding was blocked using 2% bovine serum albumin, 1% Triton X-100 for 1 h at room temperature, and incubated with the affinity-purified anti-CMF1 antibody overnight at 4 °C. After extensive washing with 1× phosphate-buffered saline, donkey anti-rabbit IgG or donkey anti-mouse IgG conjugated to Cy2 or Cy3 (Jackson Laboratory) was incubated at room temperature for 1 h. Affinity-purified anti-CMF1 was incubated overnight at 4 °C. After extensive washing in 1× phosphate-buffered saline, donkey anti-rabbit IgG or donkey anti-mouse IgG conjugated to Cy2 or Cy3 (Jackson Laboratory) was incubated at room temperature for 1 h and washed extensively in phosphate-buffered saline. These samples were then visualized using epifluorescence or confocal microscopy.

Peptide competitions (100 molar excess of immune peptide) were performed for Western blot and immunofluorescence analyses to ensure antibody specificity. This treatment completely abolished antibody binding in both assays (see Fig. 4A as an example).

RT-PCR—Staged embryonic tissues and cultured cardiogenic mesendoderm were collected, and total RNA was isolated using the Trizol reagent per the manufacturer's instructions (Life Technologies, Inc.). The Access RT-PCR system (Promega) was used to amplify cDNA from 100 ng of total RNA. Primers used to amplify CMF1 are 5′-GAGTT-GAATCTGAGAAGCGACACACC-3′ and 5′-CACCTTTTCTGGAGACCCTC-3′. vMHC primers are 5′-GCTAAACACACCAAGCAG-3′ and 5′-CTTTATATCTGGAGGCCAGG-3′. GAPDH primers are 5′-GGGTCTT-ATGACACTGTCC-3′ and 5′-GTAAGCTTCCATCAGCTG-3′. NKK-2.5 was amplified using primers designed by Schultheiss et al. (11). Conditions for amplification were: 48 °C, 45 min; [94 °C, 0.5 min; 53 °C, 0.75 min; 68 °C, 1 min]×25; and 68 °C, 7 min. Negative controls including no template and no reverse transcriptase did not produce an amplification product under these conditions. Primer concentration, RNA input, and cycle numbers were optimized to ensure amplification in the linear range.

Southern Blot Analysis—Chicken genomic DNA was digested over night at 37 °C with each of the following restriction endonucleases: EcoRI, HindIII, BamHI, and PstI (Promega). This DNA was then electrophoresed in an 0.8% agarose gel and visualized with ethidium bromide. DNA was then transferred via capillary action to a nylon membrane and UV-cross-linked (Stratagene). The blot was prehybridized in RapidHyb (Amersham Pharmacia Biotech) for 1 h at 65 °C and hybridized overnight with radiolabeled chicken pCMF1.29a.1 probe containing sequences for the carboxyl terminus of CMF1 at 65 °C. Blots were washed three times in 2× SSC, 0.1% SDS for 1 h at 65 °C. When corresponding murine LEK1 probe (pLEK1.29a.1) was used, hybridization was performed at 55 °C, and the blot was washed three times in 2× SSC; 0.1% SDS for 1 h at 60 °C. After the specified washes, blots were exposed using Biomax intensifying screen and Biomax film (Kodak).

RESULTS

Avian CMF1 Is Related to the Mammalian Proteins CENP-F, Mitosin, and LEK1—Sequence analyses indicate that CMF1 is related to an emerging family of nucleoproteins that include CENP-F (13), mitosin (14), and LEK1 (15). The predicted chicken CMF1 protein is 65% similar to human CENP-F/mitosin and mouse LEK1. An additional unifying characteristic of these proteins is that approximately 40% of their amino acid composition is leucine, glutamic acid, or lysine. These proteins are also predicted to have a similar secondary structure that is composed mainly of α-helices separated by loops and a globular carboxyl terminus. In addition, this novel protein family has a conserved collagenary array of potential regulatory domains. All domains depicted in Fig. 1 are conserved in all proteins. Similar to CENP-F, mitosin, and LEK1, CMF1 contains multiple putative leucine zippers located throughout its length. A carboxyl-terminal bipartite nuclear localization signal (NLS) that has been shown to be functional in the mammalian proteins is also present within CMF1. The isolated NLS of CMF1 can direct the efficient nuclear transport of a reporter protein.2 Additionally, to the NLS, all four proteins have an atypical Rb binding site. Ligand screening and fusion protein-binding assays have shown that mitosin can interact with Rb using this atypical domain (14).

Similarities in amino acid composition and conservation of putative regulatory domains raise the possibility that CMF1, LEK1, CENP-F, and mitosin are related members of a gene family. To determine whether related sequences were present in the avian genome, chicken genomic blots were probed with a

2 E. Dees, L. M. Pabón-Peña, R. L. Goodwin, and D. Bader, submitted for publication.
Probing with CMF1 at high stringency revealed a single hybridizing band. When the same blot was reprobed with LEK1 at lower stringency (see "Experimental Procedures"), multiple hybridizing bands were detected. With the exception of the CMF1-positive band, these additional bands did not hybridize with LEK1.

Figure 2. Southern blot analysis of chicken genomic DNA with CMF1 and LEK1. Chicken genomic DNA was cut with the denoted restriction endonucleases and blotted using standard methods with CMF1 and LEK1. Chicken genomic DNA was cut with the denoted restriction endonucleases and blotted using standard methods with CMF1 and LEK1.

FIG. 3. Analysis of the CMF1 protein in developing chicken embryos. A, Western blot analysis was performed on stage 15 chicken embryonic hearts. Lane 1 shows the protein product detected by the affinity-purified CMF1 polyclonal antibody (Ab). Lane 2 shows that anti-CMF1 antibody activity is abolished after competition with the immune peptide (100 nmol excess). In lane 3, the same protein extract was probed with MF20, showing the relative migration of sarcomeric MHC. B, Western blot analysis detects the cardiac-specific expression of CMF1 protein in stage 15 chicken embryos. Lanes 1 and 2 show the anterior and posterior regions, respectively, of the embryo. Lane 3 shows the CMF1 protein detected in the heart. C, a transverse section of a stage 13 chicken thorax shows the heart-specific expression of CMF1. Phase (a) and fluorescence (b) images demonstrate that CMF1 protein is expressed at high levels in the heart tube.

is confined to myocytes.

CMF1 Has a Tissue-restricted Protein Expression—LEK1, CENP-F, and mitosin are observed in a wide variety of cells. Nevertheless, only the expression pattern and subcellular localization of LEK1 in embryos have been reported (15). To better understand CMF1 function during embryogenesis, we characterized the expression of CMF1 protein. Western blot analyses using hearts of stage 15 chicken embryos show that CMF1 is significantly larger than the 200-kDa MHC protein (Fig. 3A, lanes 1 and 3, respectively). The predicted size of CENP-F/mitosin/LEK1 proteins is approximately 350 kDa. Competition analysis with the immune peptide shows that the band detected in the Western blots is specific to this CMF1 antibody (Fig. 3A, lane 2). Furthermore, the expression of various CMF1 constructs in bacterial or eukaryotic cells demonstrates that this antibody specifically detects a product derived from the CMF1 transcript (data not shown).

To determine the spatial distribution of CMF1 protein, we analyzed various regions of stage 15 chicken embryos. Western blot analyses show that although CMF1 protein is expressed at high levels in the heart, it is not detected in the anterior or posterior regions of early chicken embryos (Fig. 3B). Immunofluorescence analyses were performed to characterize the cellular distribution of CMF1 expression in developing embryos. A transverse section of a stage 13 chicken thorax demonstrates that CMF1 protein expression is restricted to the heart (Fig. 3C). Phase and immunofluorescence analyses of a day 7 chicken heart demonstrate that CMF1 expression is absent in epicardial cells but is present in myogenic cells (Fig. 4, A and B). Double immunostaining with the CMF1 antibody and the molecular marker of quail endothelial cells QH1, demonstrates that CMF1 is absent from endothelial cells within developing vessels and the endocardium (Fig. 4C). Cardiac expression of CMF1 is detected throughout embryogenesis; however, it significantly decreases after day 7 (Fig. 5 and data not shown). These data suggest that, unlike CENP-F, mitosin, and LEK1, with their broad expression patterns, CMF1 protein expression...
CMF1 Is Expressed prior to the Initiation of Expression of Structural Muscle Genes—Disruption of MHC expression as a result of the retrovirally mediated inhibition of CMF1 function in cardiogenic mesoderm indicated that this novel protein had a regulative role in cardiac myogenesis (1). If CMF1 were to have a regulative role in the initial steps of heart development, its mRNA and/or protein products should be expressed prior to the initiation of expression of structural muscle genes. To characterize the temporal expression pattern of CMF1 mRNA during early chicken embryogenesis, we performed RT-PCR analyses using RNAs between stages 4 and 15. In these experiments, the expression of CMF1 is detected as early as stages 4–5 (Fig. 5). During this time frame, progenitor cells are committed to the cardiac myogenic lineage but have not differentiated into myocytes. The onset of cardiac myogenic differentiation is marked by the expression of structural genes such as vMHC (6, 8, 11). A comparison of CMF1 and vMHC reactions in Fig. 5 shows that the expression of CMF1 is detected prior to the initiation of vMHC expression. The consistent levels of GAPDH demonstrate that equivalent amounts of RNA were utilized in the embryonic stages analyzed. It should be noted that the CMF1 transcript can be detected by RT-PCR in non-cardiogenic tissues (data not shown). Therefore, analysis of CMF1 protein expression was conducted during these early stages.

We examined the expression pattern of CMF1 protein with reference to cardiac myogenesis, using the expression of sarcomeric MHC as a marker of differentiation. Cardiogenic mesoderm or hearts from stages 6–14 and day 11 embryos were isolated and processed for Western blot analysis. As seen in Fig. 5B, CMF1 is first detected in stage 6 cardiogenic mesoderm and is maintained during the early stages of heart development. The level of CMF1 expression falls after the first week of development (Fig. 5B). In contrast, expression of sarcomeric MHC is first detected at stage 10 and is maintained throughout embryogenesis. It should be noted that Han et al. (7) detected MHC protein expression in a minor group of cells flanking the anterior intestinal portal at stage 8 using immunofluorescence analysis. Our Western analyses included the entire cardiogenic field and did not detect the expression of MHC in this group of cells. The present data show that CMF1 protein is detected prior to cardiomyogenic differentiation. To determine the cellular distribution of CMF1 protein prior to the differentiation of cardiac myocytes, we conducted immunofluorescence analysis of stage 5 embryos. As seen in Fig. 5C, CMF1 protein is observed at high levels in the anterior mesoderm (panels a–c); however, it is absent from posterior mesoderm (panels d–f). CMF1 protein distribution is broad within the anterior mesoderm, extending from midline laterally at this stage. At this early embryonic stage, we cannot determine whether all CMF1-positive cells are cardiac precursors. The implications of these observations are discussed below (see “Discussion”).

CMF1 Protein Expression Precedes the Appearance of Sarcomeric MHC Protein in the Heart along the Anteroposterior Axis—CMF1 protein is detected in the mesoderm prior to the differentiation of cardiac myocytes. However, the previous data do not determine the relationship of CMF1 protein expression relative to the expression of structural proteins in the heart. To determine the pattern of CMF1 protein expression during the initial phases of cardiac myogenic differentiation, stage 10 hearts were serially sectioned and reacted with anti-CMF1 and anti-MHC for double immunofluorescence analysis. At this point in development, lateral anterior splanchic mesoderm has fused to form a single heart tube. Posteriorly, the paired heart tubes have not yet fused (diagrammed in Fig. 6A). Cardiomyogenesis, including the expression of muscle-specific genes and their protein products, proceeds along the anteroposterior axis within cardiogenic mesoderm (5, 7, 8, 21). An analysis of serial sections with MF20 reveals the anteroposterior wave of myogenic differentiation from the fused heart tube (Fig. 6B, d) into the unfused, paired heart tubes (Fig. 6B, h, and at minor levels in 6B, i). The posterior regions of the unfused tubes are not positive for MHC (Fig. 6B, p). Later, all myogenic cells of the heart tubes express muscle-specific structural proteins. Interestingly, examination of the same sections demonstrated that CMF1 protein is expressed throughout the fused and unfused heart tubes (Fig. 6B, c, k, and o). Taken together, these data demonstrate that CMF1 protein expression precedes that of muscle-specific protein expression in a temporal and spatial manner.

The Early Embryonic Expression of CMF1 Is Independent of the BMP Signaling Pathway—Our previous data demonstrated that disruption of CMF1 function blocks cardiomyogenic differentiation (1). Schultheiss et al. (11) have shown that NKK-2.5 expression, which is critical for the differentiation of avian cardiac myocytes, is dependent upon BMP signaling. To determine whether CMF1 expression is regulated in the same BMP-dependent manner as NKK-2.5, cardiogenic mesoderm and associated endoderm were isolated, cultured in the presence or absence of the BMP antagonist noggin (12), and assayed for cardiomyogenic differentiation using RT-PCR and immunochromical analyses. RT-PCR analyses showed that control cultures expressed high levels of NKK-2.5, CMF1, and vMHC (Fig. 7A). As previously reported by Schultheiss et al. (11), noggin blocked the expression of NKK-2.5 and the downstream gene product vMHC in the anterior mesoderm. Interestingly, noggin treatment did not block the expression of CMF1 in these same cultures when assayed by RT-PCR (Fig. 7A). The sensitivity of RT-PCR assays raises the possibility that the CMF1 RNA detected after noggin treatment is derived from a minor non-cardiogenic component of the embryonic explants. To address the possibility that only non-cardiogenic cells express CMF1 following noggin treatment, we analyzed cardiogenic mesendoderm explants at the cellular level using the CMF1 antibody (Fig. 7B). Stage 4 anterior mesendoderm cultured for 24 h were reacted with anti-MHC and anti-CMF1. As seen in
Fig. 5. Analysis of CMF1 expression during early cardiogenesis. A, RT-PCR was performed using RNAs from stage 4–15 cardiogenic tissues with CMF1, vMHC, and GAPDH primers. CMF1 is detected prior to the onset of vMHC expression. B, Western blot analysis of stage 6, 8, 10, 14 and day 11 heart. Equivalent amounts of protein at each stage were analyzed for sarcomeric MHC and CMF1. CMF1 is detected at stages 6–14 but is absent at day 11. MHC is detected by MF20 beginning at stage 10.

C, immunohistochemical analysis of stage 5 embryos. Panels a–c show a transverse section through the anterior portion of a stage 5 embryo (a, phase; b, anti-CMF1; c, YoPro; double arrows, ectoderm; single arrows, endoderm). Panels d–f show a transverse section through the posterior portion of a stage 5 embryo (d, phase; e, anti-CMF1; f, YoPro; double arrows, epiblast; single arrows, endoderm). The diagram of a stage 5 embryo in the lower right panel illustrates the relative positions of the sections analyzed in C.

Fig. 6. Analysis of CMF1 expression in the stage 10 heart. A, 8-μm serial sections were cut through the entire stage 10 heart. The approximate positions (numbered 1–4) of the sections along the anteroposterior axis, shown in B, are given. B, position 1 is at the level of the fused heart tube and has strong staining of anti-CMF1 and anti-MHC. Positions 2–4 are increasingly posterior levels in the unfused tubes, showing decreasing levels of anti-MHC staining and nearly constant levels of anti-CMF1. Phase, 4,6-diamidino-2-phenylindole (DAPI), anti-CMF1, and anti-MHC images are shown for each section. Ab, antibody.

Fig. 7. CMF1 expression is independent of BMP signaling. A, RT-PCR analysis was used to detect the presence of CMF1, NKX-2.5, vMHC, and GAPDH in control and noggin-treated cultures. Although noggin blocks expression of NKX-2.5 and vMHC, CMF1 expression is not inhibited by this BMP-antagonist. B, immunohistochemical analysis of stage 4 cardiac mesendoderm grown for 24 h in the absence (a–d) or presence (e–h) of noggin. Phase (a and e), 4,6-diamidino-2-phenylindole (DAPI, b and f), anti-CMF1 (c and g), and anti-MHC (d and h) show positive CMF1 staining in both situations, whereas anti-MHC staining is greatly diminished with noggin treatment. At stage 4 mesendoderm was grown in the presence of noggin, cultures remained positive for anti-CMF1, but anti-MHC staining was greatly reduced (Fig. 7B, e–h). A similar pattern was observed in control and experimental cultures of stage 6 mesendoderm, although more MF20-positive cells were observed (data not shown). There are many non-CMF1-positive cells present in both the control and experimental cultures. These data suggest that BMP signaling is not required for CMF1 RNA and protein expression. In addition, these immu-
nochemical analyses suggest that cells expressing CMF1 following noggin treatment are not likely to be exclusively non-cardiogenic cells, as CMF1 colocalizes with MHC-expressing cells in control cultures. These experiments also show that CMF1 is not sufficient to activate NKK-2.5 expression and cannot direct cardiogenic differentiation in the absence of factors such as NKK-2.5.

**DISCUSSION**

**CMF1 Is a Member of an Emerging Family of Proteins—** CMF1 is related to a recently identified family of proteins characterized by their large size and by an abundance of α-helices with intervening loops and conserved regulatory domains. CENP-F and mitosin have been isolated from HeLa cells by two independent laboratories (13, 14). Recently, our laboratory has identified a related protein, LEK1, that is ubiquitously expressed in early murine embryos (15). LEK1 is approximately 80% homologous to CENP-F and mitosin. Here we show that avian CMF1 has a 65% amino acid similarity to human CENP-F, mitosin, and murine LEK1. In addition, CMF1 has the conserved collinear array of predicted functional domains characteristic of this family. Multiple leucine zippers are present in the same relative positions within each molecule. Li et al. (24) have used yeast two-hybrid analysis to demonstrate the potential of these zippers to homo- and heterodimerize, suggesting that protein-protein interactions may be regulated via these conserved domains. This novel protein family also has a conserved atypical Rb-binding site. Mitosin was first identified by its ability to bind Rb, and further analyses have shown that this atypical Rb-binding domain mediates the interaction between these two proteins (14). Although their precise function is unresolved, disruption of any of these proteins leads to an alteration of cell division and/or differentiation of specific cell lineages. Although significant variation in expression and potential function exists, the similarities in amino acid composition, overall structural homology, and conservation of regulatory protein motifs have led us to hypothesize that CMF1 is a member of the emerging CENP-F/mitosin/LEK1 family of proteins.

**CMF1 Is a Novel Member within the CENP-F/Mitosin/LEK1 Family—** Sequence conservation and structural homology predict that CMF1 is related to CENP-F, mitosin, and LEK1. Nevertheless, CMF1 appears to be distinct from other members of this emerging family. First, the protein expression pattern of CMF1 in the embryo is different from that of the mammalian proteins. Murine LEK1, which is more closely related to CENP-F and mitosin, is ubiquitously expressed in the early mouse embryo (15). Although analysis of CENP-F and mitosin in developing embryos has not been reported, they are both expressed in many non-myogenic cell lines. In contrast, our current data show that CMF1 protein has a restricted expression pattern. While an immunologically related protein is expressed in skeletal muscle cells, it is clear that CMF1 does not have the broad pattern of protein expression observed for LEK1, CENP-F, and mitosin. It should be noted that RT-PCR analysis has detected CMF1 transcripts in non-cardiogenic tissues (data not shown). Nevertheless, the overall distribution of CMF1 protein varies significantly from that of the related mammalian proteins.

Another major difference between CMF1 and other family members is in the subcellular distribution. A bipartite nuclear localization domain is present in the carboxyl terminus of all four proteins. This bipartite NLS has been shown to be functional in all three mammalian proteins, and transfection analyses with the isolated CMF1 NLS also demonstrate that it directs the efficient transport of a reporter protein to the nucleus. However, in this report we show that CMF1 localizes in the cytoplasm of differentiated myocytes even at the earliest stages of cardiac differentiation (Fig. 6). This cytoplasmic distribution raises the possibility that the NLS is not functional in the context of additional sequences and that CMF1 is a non-nuclear member of this family. It is known that mitosin can form dimers (25). It is possible that CMF1 may interact with other members of this family to regulate nuclear/cytoplasmic localization. Finally, different regions of CMF1 may have distinct subcellular localizations. We have evidence that LEK1 is proteolytically cleaved and that the NLS-containing C-terminal region is translocated to the nucleus. It is possible that CMF1 undergoes a similar proteolytic cleavage and that its carboxyl terminus, containing the NLS domain, localizes to the nucleus. CMF1 antibodies directed against the carboxyl-terminal domain will have to be developed to test this hypothesis.

Another difference from CENP-F and mitosin is that the expression of CMF1 is not cell cycle-regulated. CENP-F and mitosin accumulate at the end of G1 phase and are quickly degraded at the end of telophase (13, 14). In contrast, CMF1 appears to have a cell cycle-independent expression pattern in vivo. In the avian heart, all myocytes are initially mitotically active, whereas later only subepicardial myocytes remain proliferative (Refs. 26 and 27 and footnote 4). Myocytes located in the trabeculae of the ventricle have exited the cell cycle and are essentially in G0. CMF1 protein is present in all of these cells, and thus mitotic and non-mitotic myogenic cells contain CMF1 protein (Fig. 4D). Although the subcellular distribution of LEK1 varies from that of CMF1, its expression is also cell-cycle independent (15). These data suggest a dynamic diversity in the localization and accumulation of these proteins during the mitotic cycle.

Finally, the functions of these proteins appear to vary. Initial functional analyses indicate that CENP-F, mitosin, and LEK1 regulate or are fundamentally involved in the general process of cell division. The introduction of dominant-negative forms of these three proteins into cell lines alters the pattern of mitosis, generally slowing down or inhibiting the process (14, 15). Their potential effects on cell differentiation are less well understood. The disruption of CMF1 function inhibits MHC expression in differentiating cardiac mesoderm (1), suggesting that CMF1 may play a role in the regulation of cardiomyogenic differentiation. Although the present data detect the central portion of CMF1 only in the cytoplasm, and mechanisms for transient translocation of the C terminus to the nucleus are proposed above, CMF1 may act in the cytoplasm to influence muscle protein expression. This type of regulatory mechanism has also been proposed for the intermediate filament protein desmin (28, 29). Experiments supporting this alternative show that the abrogation of desmin in murine embryonic stem cells inhibits the expression of muscle markers such as MyoD, myogenin, and MHC. Furthermore, non-nuclear factors such as AKAP75 have been shown to significantly influence transcriptional activation by modulating type II protein kinase A availability (30). It is possible that CMF1 can regulate the availability of factors required for differentiation via their interaction with leucine zipper domains.

The pattern of CMF1 expression and the diversity in its subcellular localization suggest a restricted function in the developing embryo. Still, the work of others and our own work suggest that CENP-F/mitosin/LEK1 play an essential role in mitosis in a multitude of cell types derived from all three germ layers (13–15). Although CMF1 and related protein are expressed in developing striated muscle, CENP-F, mitosin, and LEK1 are broadly expressed. Thus, one would suspect that

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3 L. M. Pabón-Peña, R. L. Goodwin, L. J. Cise, and D. Bader, manuscript in preparation.
4 R. Thompson, personal communication.
other proteins of this class are expressed in other cells of the avian embryo. Indeed, chicken genomic blots probed with the conserved 3’ coding sequences of CMF1 and LEK1 suggest that other related genes are present in the avian genome (Fig. 2 and data not shown). It is possible that members of this gene family have different patterns of expression and interact alone or together to participate and/or regulate the mitotic process in diverse developmental settings. The data presented in this report, in conjunction with the previously reported effects of CMF1 on heart development and the effects of the mammalian proteins on cell proliferation, suggest that this novel protein family could provide developing cells with a mechanism that interfaces cell division and differentiation.

**CMF1 Is Expressed prior to the Activation of Cardiac Structural Genes**—Our previous results show that disruption of CMF1 function blocks the expression and/or accumulation of sarcomeric MHCs (1). If CMF1 were to play a regulative role in differentiation, it should be expressed prior to the onset of structural gene activation. The current data show that CMF1 mRNA and protein are expressed prior to sarcomeric MHC. Western blot analysis demonstrates that CMF1 is present at stage 6 (Fig. 5). Immunohistochemical analyses of stage 5 embryos show that CMF1 protein is detected at high levels in anterior mesodermal cells, but it is absent from posterior mesodermal cells. CMF1 expression at this stage extends from midline to the lateral portions of the anterior mesoderm. Although later CMF1-positive cells derived from anterior lateral mesoderm are restricted to cardiac myocytes, the current data cannot determine whether all CMF1-positive cells at stage 5 are cardiac precursors. It is possible that CMF1 protein expression is not confined to cardiac precursors at this stage. Indeed, it is well documented that other known regulators of cardiac development may have broader expression patterns early in embryogenesis, whereas later they become highly enriched or restricted to cardiac cells (11, 12, 31–33). A second possibility is that cardiogenic mesoderm are widely distributed anterior mesoderm at this stage of development. Previous studies have shown that cardiac precursors move from midline to assume positions in lateral splanchic mesoderm (2). Ehman and Yutzey (21) have recently reported that cardiogenic cells migrate to the lateral most portion of anterior mesoderm, whereas other studies have suggested that these progenitors are more medially positioned (22, 34, 35). Indeed, when medial or lateral anterior mesoderm is cultured in vitro, cardiac myocytes readily differentiate from cell populations (compare fate maps, 22 (Fig. 1) and 21 (Fig. 7)). Thus, it may be that cardiac progenitors are more widely distributed than previously thought and/or that progenitors are still migratory within developing mesoderm at this time. Our data cannot distinguish between the two possibilities stated above.

Although the precise location of cardiac progenitors and molecular regulation of commitment remain unresolved, the stage 10 heart can be used to directly assay that factors are expressed prior to the activation and/or accumulation of structural proteins. Previous studies have shown that myogenic differentiation as defined by the expression of structural gene products occurs as a wave along the anteroposterior axis (5, 7, 8, 21). Clearly, NKK-2.5, GATA, and HAND gene products are expressed in the heart tube prior to the activation of structural genes. Our current data show that the wave of CMF1 protein expression precedes differentiation of cardiogenic mesoderm along the anteroposterior axis of the developing heart. These data along with previous studies on the disruption of CMF1 function suggest that this protein plays a role in the regulation of cardiac myogenesis.

**CMF1 as a BMP-independent Regulator of Cardiac Muscle Development**—Our current data show that the expression of CMF1 protein precedes the activation of cardiac structural gene products. Previous data demonstrate that disruption of CMF1 function inhibits the expression of structural muscle proteins such as MHC, and thus CMF1 appears to be necessary for cardiac differentiation. These data along with the current study showing the restricted expression of the CMF1 protein in myogenic cells suggest that CMF1 plays a role in the regulation of cardiomyogenic differentiation. The activation of cardiac differentiation is regulated by the interaction between anterior mesoderm and endoderm (11, 36). Expression of NKK-2.5, which is thought to be mediated by endodermally derived BMPs, is essential for the activation of downstream structural genes such as vMHC and the differentiation of cardiac myocytes (11, 12). The current study shows the continued expression of CMF1 in the presence of the BMP antagonist noggin and suggests that CMF1 expression is not dependent upon the BMP signaling pathway. Still, expression of NKK-2.5 and vMHC was inhibited in these same cultures (Fig. 7). The absence of muscle-specific gene expression in noggintreated cultures indicates that CMF1 expression in the absence of factors such as NKK-2.5 is not sufficient to activate terminal differentiation of cardiogenic mesoderm. Taken together, these data suggest that CMF1 may act as a critical BMP-independent component in the regulation of vertebrate cardiac myogenesis.

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