CLIF, a Novel Cycle-like Factor, Regulates the Circadian Oscillation of Plasminogen Activator Inhibitor-1 Gene Expression*

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The onset of myocardial infarction occurs frequently in the early morning, and it may partly result from circadian variation of fibrinolytic activity. Plasminogen activator inhibitor-1 activity shows a circadian oscillation and may account for the morning onset of myocardial infarction. However, the molecular mechanisms regulating this circadian oscillation remain unknown. Recent evidence indicates that basic helix-loop-helix (bHLH)/PAS domain transcription factors play a crucial role in controlling the biological clock that controls circadian rhythm. We isolated a novel bHLH/PAS protein, cycle-like factor (CLIF) from human umbilical vein endothelial cells. CLIF shares high homology with Drosophila CYCLE, one of the essential transcriptional regulators of circadian rhythm. CLIF is expressed in endothelial cells and neurons in the brain, including the suprachiasmatic nucleus, the center of the circadian clock. In endothelial cells, CLIF forms a heterodimer with CLOCK and up-regulates the PAI-1 gene through E-box sites. Furthermore, Period2 and Cryptochrome1, whose expression show a circadian oscillation in peripheral tissues, inhibit the PAI-1 promoter activation by the CLOCK:CLIF heterodimer. These results suggest that CLIF regulates the circadian oscillation of PAI-1 gene expression in endothelial cells. In addition, the results potentially provide a molecular basis for the morning onset of myocardial infarction.

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Experimental Procedures

Construction of Plasmids—The endothelial PAS domain protein 1 (EPAS1) bait plasmid pBDGAL4-EPAS1 was constructed by amplification of cDNA encoding amino acids (aa) 30–380 of the human EPAS1 protein by the polymerase chain reaction (PCR). The amplified fragment was cloned into the vector pBDGAL4 Cam (Stratagene). The plasmids for GAL4 DNA binding domain fusion proteins were constructed by amplification of cDNAs encoding bHLH/PAS domain (ARNT, aa 70–508; BMAL1, aa 64–445; CLIF, aa 74–445) by PCR. The

activity. PAI-1 activity shows a clear circadian oscillation peaking in the early morning, and this may account for the morning onset of myocardial infarction (1, 2). However, the molecular mechanisms regulating this circadian oscillation remain unknown.

Every organism from bacteria to humans has an internal biological clock that adapts its activity to a circadian rhythm. Recently, the molecular mechanisms underlying these circadian processes are beginning to be elucidated (3–5). The biological clock is composed of transcriptional-translational feedback loops (3). Many of these components, including CLOCK, CYCLE, and PERIOD (PER), belong to the basic helix-loop-helix (bHLH)/PAS domain family of transcription factors. Thus, the PAS domain plays a crucial role in regulating the biological clock (4). In mammals, CLOCK and BMAL1, the mammalian counterpart of Drosophila CYCLE, induce Per and Cryptochrome (Cry) gene expression (6). The PER and CRY proteins in turn act as negative components of the feedback loop by suppressing CLOCK:BMAL1-mediated transcription through CACGTG E-box enhancer elements (7, 8).

The central circadian pacemaker in mammals is localized in the hypothalamic suprachiasmatic nucleus (SCN) (4). Recent studies have shown circadian oscillations of various transcripts in both peripheral tissues and cultured cells. Their underlying rhythm is inferred to be due to the same mechanisms that are present in the SCN (9–11). These peripheral clocks are synchronized with the central clock by yet unidentified humoral factors (5, 10, 12–14). The functional significance of the mammalian peripheral clocks is unknown.

We report here the identification of a novel bHLH/PAS domain transcription factor, CLIF (cycle like factor) that shares high homology with Drosophila CYCLE. Since CLIF is expressed in vascular endothelial cells, we hypothesize that CLIF may regulate the circadian oscillation of PAI-1 gene expression in endothelial cells. We show that CLIF forms a heterodimer with CLOCK and in endothelial cells up-regulates the PAI-1 gene through its E-box sites. Furthermore, PER2 and CRY1, whose expression show a circadian oscillation in peripheral tissues, inhibit the PAI-1 promoter activation by the CLOCK:CLIF heterodimer. This may account for the circadian oscillation of PAI-1 gene expression.

**EXPERIMENTAL PROCEDURES**

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amplified fragments were cloned into pBDGAL4Cam. The plasmids for GAL4 activation domain fusion proteins were constructed by PCR amplification of cDNAs encoding EPAS1 or CLOCK. The amplified fragments were cloned into the yeast GAL4 activation domain vector, pAD-GAL4 (Stratagene). The pHCLIF expression plasmid was constructed by PCR amplification of the full-length human CLIF cDNA using the primers 5'-TGAGAATTCACAGTGCTTCGACGGCTGGACTG-3' and 5'-CAAGGATCCGAGGGTCCACTGGATGTCACT-3'. The amplified fragment was digested with EcoRI and BamHI and cloned in frame into pCDNA3.1(-)... (Invitrogen). The mammalian expression vector for BMAL1 was made by PCR amplification of full-length BMAL1 and cloned into pCDNA3 (Invitrogen). The mammalian expression vectors for ARNT, CLOCK, PER2, and CRY1 are described elsewhere (6, 8, 15). The human PAI-1 promoter/luciferase reporter plasmids, p800ULUC, p549LUC, and p157LUC, were gifts from David J. Loskutoff (16). E-boxes of the PAI-1 promoter were mutated at bp -677 to -672 (m1, CACGTT to GCTAGT) and at bp -562 to -557 (m2, CACGTT to TCAGCT) using the QuickChange site-directed mutagenesis kit (Stratagene).

The authenticity of all constructs was verified by dideoxy chain termination sequencing.

**Yeast Two-hybrid Screening**—A human umbilical vein endothelial cell (HUEVC) cDNA library was constructed by cloning HUEVC cDNAs into the vector Hyb2ZAP-2.1 (Stratagene). The yeast strain YRG-2 (Stratagene) was made competent by the lithium acetate method (17).

Approximately 10^10 yeast cells were transformed with 100 μg of expression constructs using Lipo-For (Promega). Luciferase activity and ß-galactosidase activity in yeast (data not shown). To examine the expression pattern of CLIF, we performed Northern blot analysis on RNA isolated from human tissues. The human CLIF probe hybridized to four mRNAs of 8, 6, 2.4 and 2.2 kilobases. CLIF is expressed most abundantly in the brain and placenta, with lower expression in the heart, thymus, kidney, liver, and lung (Fig. 2A). In contrast to the restricted tissue distribution of CLIF, ARNT and BMAL1 were expressed in many tissues, including the brain, heart, and skeletal muscles as reported previously (Fig. 2A) (26, 27).

We further performed in situ hybridization on human tissues to identify the cellular expression of CLIF. CLIF was expressed in endothelial cells and neurons in the brain, including the SCN, i.e. the center of the circadian clock (Fig. 2B). We also detected CLIF mRNA transcripts in endothelial cells in the heart, lung, and kidney (data not shown). Additional analysis by gel mobility shift assays demonstrated that CLIF forms a heterodimer with CLOCK and bound to the E-box within the Per1 promoter (data not shown). Furthermore, transient transfection of CLOCK and CLIF together transactivated the Per-1 promoter to the same extent as CLOCK and BMAL1 (data not shown) (6). Taken together, CLIF as a heterodimerization partner of CLOCK may contribute to the control of circadian rhythm in both the central and peripheral clocks.
The endothelial cell regulation of vascular tone and fibrinolytic activity shows circadian variation (28, 29). For instance, the frequent onset of myocardial infarction during the early morning may partly result from the circadian variation of fibrinolytic activity (2, 30). In the basal state, PAI-1 is produced mainly in endothelial cells, with the highest activity in the early morning. This elevated PAI-1 activity corresponds with the frequent onset of myocardial infarction during the early morning, which may partly result from the circadian variation of fibrinolytic activity (2, 30). For instance, PAI-1 mRNA levels exhibited circadian variation. Indeed, Northern blot analysis of the mouse heart (Fig. 3A), kidney (Fig. 3B), brain, and lung (data not shown) showed circadian variation of PAI-1 mRNA levels with peak expression in the evening. This circadian oscillation pattern of mouse PAI-1 mRNA is antiphase to that of human PAI-1 activity, potentially due to the fact that rodents are nocturnal whereas humans are diurnal. To our knowledge, this is the first demonstration that the circadian oscillation of PAI-1 activity is regulated at mRNA level. The blots were then reprobed to analyze CLIF and BMAL1 expression in relation to PAI-1. These studies revealed that CLIF mRNA was constitutively expressed, whereas BMAL1 mRNA levels oscillated as reported previously (9) (Fig. 3C). The constant circadian expression pattern of CLIF is similar to that of Drosophila CYCLE (25). Per2 and Cry1 are important negative regulators of the biological clocks (32). Per2 and Cry1 expression followed a clear circadian oscillation (Fig. 3C). In these experiments, Clock was constitutively expressed as reported previously (data not shown) (33).

Overexpression of Clock and CLIF Induce PAI-1 mRNA Expression—We next examined whether the CLOCK-CLIF heterodimer is able to increase the endogenous PAI-1 mRNA in HUVEC. Using adenovirus-mediated gene transfer, we demonstrated that overexpression of CLOCK resulted in a dose-dependent increase in PAI-1 mRNA levels relative to the control cells infected with a GFP-expressing adenovirus (Fig. 3D). Coinfection of adenovirus expressing CLIF with CLOCK further increased the PAI-1 mRNA levels (Fig. 3D).

CLOCK and CLIF Transactivates the PAI-1 Promoter through the E-box Sites—To further elucidate the mechanisms by which CLOCK and CLIF increase PAI-1 mRNA levels, we...
CLOCK and CLIF transactivate the PAI-1 promoter through two E-box sites. A, CLOCK and CLIF transactivate the PAI-1 promoter. The indicated expression plasmids (0.5 µg each) were cotransfected into BAEC with the PAI-1 promoter (−800 bp)/luciferase reporter plasmid. B, mutation of the E-boxes abolishes the transactivation of the PAI-1 promoter. The E-box at bp −677 to −672 was mutated from CACGTG to GCCTAG (m1), and the E-box at bp −562 to −557 was mutated from CACGTG to TGCTGC (m2). Deletion series of PAI-1 promoter (−549 and −187) or mutated PAI-1 promoter (m1, m2, and m1+2) reporter plasmids were transfected with the indicated expression plasmids. C, binding of CLOCK:CLIF heterodimer to the E-box of the PAI-1 gene. Gel mobility shift assays were performed as described under “Experimental Procedures” using the indicated in vitro translated proteins and a 32-bp double-stranded oligonucleotide (5′-CTGGA-CACGTGGGGAGACAATCACGTGGCTGG-3′) probe containing the two E-boxes derived from the sequence of the PAI-1 promoter. Binding experiments were also performed in the presence of unlabeled E-box consensus oligonucleotide (E-Box) or unrelated nonspecific oligonucleotide (NS) at 100-fold molar excess. D, PER2 or CRY1 inhibits transactivation of the PAI-1 promoter by CLOCK:CLIF. The indicated expression plasmids (0.3 µg each) were cotransfected into BAEC with the PAI-1/luciferase reporter plasmid. A, B, and D, -fold induction represents the ratio (mean ± S.E.) of luciferase activity in cells transfected with expression plasmid to that in cells transfected with empty vector (pcDNA3).

PER and CRY function by inhibiting CLOCK:BMAL1-mediated transcription of their own promoter through CACGTG E-box enhancer elements (7, 8). We found that Per2 and Cry1 mRNAs showed a clear circadian oscillation in peripheral tissues (Fig. 3B). Therefore, we asked whether PER2 or CRY1 could affect the CLOCK:CLIF activation of PAI-1. Cotransfection of PER2 partially inhibited CLOCK:CLIF-dependent transcription, while cotransfection of CRY1 abolished the induction of the PAI-1 promoter (Fig. 4D). These data suggest that PER2 and/or CRY1 suppress the CLOCK:CLIF-mediated transcription of PAI-1, resulting in the circadian oscillation of PAI-1 gene expression.

We identified that both E-boxes in PAI-1 promoter are responsible for its activation by CLOCK:CLIF. It is noteworthy that the E-box at bp −677 to −672 overlaps with the sequence of the 4G/5G polymorphism of the PAI-1 promoter, which correlates with serum levels of PAI-1 and the frequency of ischemic diseases (34, 35). Thus, the 4G/5G polymorphism of PAI-1 promoter may affect the binding of CLOCK:CLIF to this E-box or interaction with adjacent transcription factors, resulting in an altered circadian expression pattern of PAI-1 activity and the 4G/5G polymorphism.
Recent data indicate that the mammalian circadian system is hierarchically organized, with self-sustained oscillators in the SCN entraining dampened oscillators in the periphery (12). However, the physiological significance of these peripheral oscillators remains unknown. Our results suggest that a peripheral tissue pacemaker regulates the circadian expression of PAI-1 gene directly. There are two heterodimers containing CLOCK; BMAL1:CLOCK dimer and CLIF:CLOCK dimer. The oscillation pattern and distribution of these two dimers are different in peripheral tissues. Therefore it may be possible that the combination of CLOCK:CLIF or CLOCK:BMAL1 together with PER and CRY function to regulate the peripheral pacemakers differently. Thus circadian rhythm in mammals as well as the SCN entraining dampened oscillators in the periphery (12).

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