Histone Acetyltransferase-dependent Chromatin Remodeling and the Vascular Clock*

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Rhythmic gene expression is central to the circadian control of physiology in mammals. Transcriptional activation of Per and Cry genes by heterodimeric bHLH-PAS proteins is a key event in the feedback loop that drives rhythmicity; however, the mechanism is not clearly understood. Here we show the transcriptional coactivators and histone acetyltransferases, p300/CBP, PCAF, and ACTR associate with the bHLH-PAS proteins, CLOCK and NPAS2, to regulate positively clock gene expression. Furthermore, Cry2 mediated repression of NPAS2-BMAL1 is overcome by overexpression of p300 in transactivation assays. Accordingly, p300 exhibits a circadian time-dependent association with NPAS2 in the vasculature, which precedes peak expression of target genes. In addition, a rhythm in core histone H3 acetylation on the mPer1 promoter in vivo correlates with the cyclical expression of their mRNAs. Temporal coactivator recruitment and HAT-dependent chromatin remodeling on the promoter of clock controlled genes in the vasculature permits the mammalian clock to orchestrate circadian gene expression.

Circadian rhythms, which are generated by cell autonomous biological clocks, allow for the appropriate temporal synchronization of physiology and behavior, optimizing the efficiency of biological systems (1, 2). In mammals, the circadian timing system is hierarchical, with the master clock located in the hypothalamic suprachiasmatic nuclei (SCN)(3). Circadian oscillators have been uncovered in both central and peripheral tissues, with the suprachiasmatic nucleus (SCN) coordinating temporal physiology by synchronizing peripheral oscillators through both neural and humoral outputs (4–8).

Transcriptional regulation is central to clock function. Pacemaker rhythms are generated and sustained by positive and negative transcriptional/translational feedback loops (9). Positive components include the bHLH-PAS proteins CLOCK and BMAL1 (also known as MOP3) driving transcription as functional heterodimers through E-box enhancer elements (CACGTG) (10). NPAS2 (also known as MOP4) is a parologue of CLOCK and behaves similarly in cell and biochemical assays (8, 11–14). We have observed a robust rhythm in NPAS2 mRNA expression in the aorta, kidney, and heart. Furthermore, NPAS2 can operate in the core feedback loop in the vasculature and forebrain (8, 13, 14). Known targets for these effector molecules are the Period (Per1-3) and Cryptochrome (Cry1-2) genes (2). As PER and CRY cytoplasmic levels rise, they can translocate back to the nucleus, and negatively regulate their own transcription by directly interacting with CLOCK:BMAL1 or NPAS2:BMAL1 heterodimers (9, 15).

Thereafter, nuclear levels of the PER/CRY complex decline, relieving repression on the bHLH-PAS heterodimer and restarting the cycle with a period of ~24 h. The positive limb of the feedback loop also involves the regulation of bmal1, which cycles robustly antiphase to Per and Cry (9). CLOCK and BMAL1 also drive the expression of the orphan nuclear receptor Rev-erba in the SCN and liver (16). The Bmal1 gene contains Rev-Erba/ROR response elements in its promoter, and its expression is repressed by Rev-Erba (16). Therefore as Per, Cry, and Rev-erba levels rise, Bmal1 levels fall. The precise regulation of circadian timing systems extends beyond RNA kinetics and includes recently identified transcriptional and post translational modifications (17, 18). For example, CLOCK, BMAL1, PER1, and PER2 all undergo temporal changes in phosphorylation in mouse liver. These affect their activity, stability, and subcellular localization (18, 19).

Covalent modifications of histones on the chromatin template, such as acetylation, phosphorylation, and methylation, influence gene specific regulation (20). The correlation between acetylation of core histones and transcriptional induction has been well established (21, 22). The association of transcription factors, recruitment of coactivator complexes, and the targeting of gene promoters to induce histone acetylation and chromatin remodeling is a sequential process, resulting in transcriptional activation. Indeed, CREB-binding protein (CBP) is known to associate directly with RNA polymerase II (23) a critical step in the rapid assembly of functional preinitiation complexes at promoters (24, 25). For example, p300/CBP-associated factor (PCAF) preferentially acetylate histone H3, while histone H3 and histone H4, are substrates for p300/CBP. Previous reports, linking light exposure during subjective day to histone H3 phosphorylation and associated SCN induction of circadian genes, strongly suggests that chromatin remodeling complexes might temporally regulate rhythmic gene expression exhibited by clock genes in the circadian feedback loop (26). Furthermore, Etchegaray et al. (27) recently demonstrated an important role for histone acetylation in regulating circadian gene expression. These studies examined the role of CLOCK: p300-dependent...
chromatin remodeling in the liver. We report that this mechanism extends to a distinct peripheral clock in the vasculature where it involves a CLOCK parologue, NPAS2 and modulates Cry-dependent repression of cyclical gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa 229 cells were grown to ~70% confluence and serum shock with 50% fetal bovine serum was carried out as described (8). Cells were harvested at indicated post-serum induction time points. Animal Handling and Tissue Harvesting—8–12-week-old c57Bl/6 wild-type male mice were synchronized to a 12:12 light/dark cycle for a period of 2 weeks before placing in DD. The animals were anesthetized at indicated time intervals and then euthanized. Tissues were harvested, immediately flash-frozen in liquid nitrogen and stored at ~70°C.

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitations (ChIP) were carried out on HeLa cells using the ChIP assay kit (Upstate Biotechnology). ChIP assays were performed on mouse tissue as described (28–30) with slight modifications. Cells were resuspended in lysis buffer containing protease inhibitors and sonicated using a Misonix Sonicator 3000 for four 15-s bursts at setting 4 for cells or eight 40-s bursts at setting 4 for tissue: this results in DNA being fragmented to sizes of 500–1000 base pairs. Immunoprecipitations were carried out using a range of antibodies: anti-acetyl Histone 3 (acetylated lysine residues 9, 14, 18, and 23), anti-acetyl lysine residues 5, 8, 12, and 16 from Upstate Biotechnology and anti-Pol II (Santa Cruz Biotechnology), which recognizes the N terminus of the large subunit of human RNA polymerase II. ChIP assays were also performed in the absence of antibody or the presence of anti-hemagglutinin (Santa Cruz Biotechnology) to control for nonspecific interactions. Immunoprecipitated DNA was analyzed by real-time PCR (ABI Prism 7000, PE Applied Biosystems).

RNA Analysis—Total RNA was extracted from cells and mouse heart tissue using RNAqueous (Ambion) and TRIzol (Invitrogen). RNA (400 ng) was used in a reverse transcription (RT) reaction (Applied Biosystems Reverse Transcription Kit). The resulting cDNA (40 ng) for each time interval was subjected to quantitative Real Time PCR.

Quantitative Real Time PCR—Primers for RNA and ChIP analysis were designed by PRIMER EXPRESS 1.0 (PE Applied Biosystems) to amplify 50–150-bp amplicons and the DNA intercalating SyBr green reagent (Applied Biosystems) was used for detection of all products. The relative differences among the intervals following serum induction for ChIP and RNA expression were calculated using the ΔΔCt method as described (Applied Biosystems User Bulletin 2). A ΔCt value was calculated for GAPDH to account for differences in starting material for RNA expression and a ΔΔCt for the ChIP total input control to normalize for variation in the amount of starting material. Primer sequences: hPer1 forward, 5′-TCTCATTGTGTCATCTATT-3′ and hPer2 reverse, 5′-CTGCCGATCGACTTTCATCATC-3′; hPer1 reverse, 5′-AGGGTTGCGCTTGTTGAA-3′ and hPer2 forward, 5′-GCCGGAATTCGCCGCTGT-3′; mPer1 forward, 5′-TGGAAACAGGAACTGACACTC-3′; mPer1 reverse, 5′-AGAATACGGGATCCTTCTG-3′; mPer2 forward, 5′-GCAGCCCGACAACATACA-3′; and mPer2 reverse, 5′-GGGACCGCGCGAGAGG-3′. Histone H3 acetylation was monitored using primers that bracket the second most conserved E-box sequence (CACGTG), the strict consensus motif for heterodimeric bHLH-PAS CLOCK/BMAL1 binding. These occur within 4 kb of the hPer1 gene promoter and each of which has been shown to contribute to the regulation of Per1 expression (31). In contrast, the hPer2 gene does not contain any such sequences within 1.6 kb of the promoter (34). Changes in mRNA levels of hPer1 and hPer2 in HeLa cells following serum induction were observed to exhibit a circadian expression pattern. Per1 levels reached a trough at T6 and then entered a cyclical expression pattern, peaking at T24 and at T48. Per2 levels, by contrast, remained high at T6, reached a measured trough at T12 exhibiting further peaks at approximately T18 and T42 (Fig. 1A). ChIP assays were performed on serum induced HeLa cells using primers that bracket the second most proximal E-box to exon 1 in the hPer1 promoter (Fig. 1B). Histone H3 acetylation peaked at T42, while histone H4 acetylation peaked at T24 and T48 which coincides with the peaks of hPer1 mRNA expression. Although we did not observe a peak in histone H3 acetylation in the first 24 h post-serum induction, the peak of histone H3 acetylation at T42 precedes that of maximum mRNA expression of hPer1 at T48. This is in agreement with our in vivo results (see below).

CBP and NPAS2 were subcloned into the GAL4BD and VP16 vectors respectively for cell-based assays. CV-1 cells were transfected with the pGM100TKLuc reporter along with combinations of GAL4, GALANPS2, VP16, and VP16CBP. Cells were harvested 48 h post-transfection and assayed for luciferase activity (Turner Design) with the pE-box reporter construct pGL3MSmLuc, pRLSV40 along with combinations of NPAS2, BMAL1, CLOCK, p300, CBP, PCAF, INHAT, ETA, TWIST, and CRY2 where indicated. The amount of DNA in each transfection was kept constant by addition of empty pCDNA3 vector. Cells were harvested 48 h post-transfection and assayed for luciferase activity by luminometer (Turner Design).

Cell Culture—HeLa 229 cells were transiently transfected as detailed (31) with the E-box reporter construct pGL3MSmLuc, pRLSV40 along with combinations of NPAS2, BMAL1, CLOCK, p300, CBP, PCAF, INHAT, ETA, TWIST, and CRY2 where indicated. The amount of DNA in each transfection was kept constant by addition of empty pCDNA3 vector. Cells were harvested 48 h post-transfection and assayed for luciferase activity by luminometer (Turner Design).

RESULTS

HAT-dependent Regulation of Vascular Circadian Rhythms

In Vivo Interaction Assays—[35]Smethionine-labeled BMAL1, CLOCK, NPAS2, ACTR, CBP, p300, and PCAF were synthesized by Promega (TNt-coupled Reticulocyte Lysate Systems), run on SDS-page gel, and expression analyzed by phosphorimager. [35]Smethionine-labeled BMAL1, CLOCK, and NPAS2 were incubated with recombinant FLAG-tagged p300 and PCAF, and immunoprecipitated with or without anti-p300 and anti-PCAF antibodies (Santa Cruz Biotechnology) or with Rabbit IgG as a control for co-immunoprecipitation assays. GST-NPAS2 was synthesized using the pGEX4T vector (American Biosciences) and used in GST pull-down assays as described (8, 31). In both assays GST-NPAS2 were run on 12% Coomassie stained SDS gel, and equal amounts were determined for pull-down experiments. Equal amounts of GST-NPAS2 and GST were incubated with [35]Smethionine-labeled ACTR, CBP, p300, and PCAF for 2 h at 4°C. The beads were washed extensively and analyzed by phosphorimager.
ACTR could interact specifically with CLOCK, BMAL1, or NPAS2. A modest interaction was observed between all three bHLH-PAS proteins and p300 (Fig. 1C, panel I), while PCAF was observed to associate preferentially with NPAS2 and, to a lesser extent, with CLOCK (Fig. 1C, panel I). Co-immunoprecipitation with anti-rabbit IgG in the presence of CLOCK and PCAF was used as an additional control (Fig. 1C, panel II).

Importantly, and in agreement with the in vitro immunoprecipitation experiments, in vitro binding assays showed formation of complexes between ACTR, p300/CBP, and PCAF and the GST-NPAS2 glutathione-Sepharose affinity matrices, (Fig. 1D). This interaction was specific since a complex was not formed between GST-NPAS2 and TAF1β (panel I). GST-NPAS2 complex from lanes 3 and 4 (panel I) were probed using anti-p300 and anti-PCAF antibodies (panel II).

Interaction of CLOCK Proteins with Coactivators in Intact Cells—The temporal colocalization of the endogenous proteins in primary cultures of human vascular smooth cells (HVSMC) was subsequently investigated. Immunofluorescent analysis of HVSMC by confocal microscopy revealed that CLOCK and p300 both exhibit predominantly nuclear localization, while NPAS2 is evident both in the nucleus and in the perinuclear cytoplasm in untreated quiescent cells (Fig. 2A). Overlay analysis of HVSMC immunostained with either anti-CLOCK or anti-NPAS2, together with anti-p300, produced discrete speckles of colocalization within the nucleus. This was more apparent for NPAS2 and p300 than for CLOCK and p300 (Fig. 2A). In addition, a Gal4BD fusion of NPAS2 and a VP16 fusion of CBP used in mammalian two-hybrid assays confirmed their association in intact cells, consistent with the GST pull-down experiments (Fig. 2B).

The association of endogenous coactivators to clock proteins was then investigated in intact cells. CLOCK and NPAS2, immunoprecipitated from quiescent HeLa cell lysates, were probed for the presence of the coactivator p300, CBP, and PCAF. p300 co-precipitated with CLOCK and, to a lesser extent, NPAS2 (Fig. 2C). However, CBP was only detected from immunoprecipitated CLOCK and not NPAS2 (Fig. 2C). Interestingly, PCAF selectively coprecipitated with only NPAS2 in these cells (Fig. 2C).

Given the precedent with CLOCK and p300 in the liver (27), we hypothesized that the NPAS:p300 interaction might exhibit temporal regulation. HeLa cells were serum shocked and ly-
sates taken at intervals following treatment. They were immunoprecipitated with Npas2 and the immune complexes were probed for p300. Though only a modest association between Npas2 and p300 was observed in quiescent cells (Fig. 2C), a robust and dynamic interaction occurred in synchronized cells (Fig. 2D, panel I). This trough was followed by a significant increase, at T20 and T24, which corresponded with maximum RNA levels of Per1 and Per2 (Fig. 1A). A low level of interaction was again evident at T36, synchronous with a fall in Per1 and Per2 mRNA levels (Fig. 2D, panel III). All data was normalized to β-actin (Fig. 2D, panel II). The observation that serum-induced cells display a rhythm in histone acetylation surrounding the Per1 promoter and that Npas2 associates in a time-dependent manner with histone acetyltransferases at a time of maximum transcriptional activity is consistent with regulation of the circadian feedback loop.

Transcriptional Activity of Npas2/Clock:BMal1 Is Modulated by Acetylation—The association of the clock bHLH-PAS proteins with the HATs, p300/CBP, PCAF and ACTR, is consistent with a role for targeted coactivator recruitment and chromatin remodeling in the temporal regulation of circadian gene activation in the mammalian feedback loop. This hypothesis was addressed by analyzing the effect of overexpression of the HAT-coactivators on E-box-induced gene transcription in intact cells. Both Npas2:Bmal1 and Clock:Bmal1 activated transcription via an E-box-dependent mechanism (10). Importantly, and consistent with previous interaction specificity assays, over expression of CBP and PCAF markedly enhanced Npas2- and Clock-mediated luciferase reporter gene activation (Fig. 3, A and B). In addition, coactivation of E-box-dependent transcription was maximally enhanced when CBP and PCAF were co-expressed (Fig. 3, A and B). Cotransfection of deletion mutants of Npas2, Npas2/H9004-322 and Npas2/H9004-425, decreased the transactivation of Bmal1 by full-length Npas2, indicating a direct effect of the coactivators on the transcription factor complex (Fig. 3C, panel II). Both of these mutants lack the LXXLL motif (Fig. 3C, panel I), which is present in nuclear receptor cofactors and in the ligand binding domains (LBDs) of some nuclear receptors, and is necessary for their interactions (37). Thus, both recruitment of specific coactivators to the clock complex and HAT-dependent activation are critical features of Clock/Npas2:BMal1-mediated E-box activation.

Furthermore, the histone acetylation regulatory proteins, E1A and Twist (32, 38) and the histone masking complex

Fig. 2. Interaction of Clock proteins with co-activators in vitro and in intact cells. A, p300, Npas2, and Clock colocalize in the nuclear regions of HVSMCs. Resting hVSMCs were incubated with anti-Clock or Npas2 and p300. Fluorescence was detected by confocal laser microscopy (B) CBP interacts with Npas2 in intact cells by mammalian two-hybrid assay. CV-1 cells were transiently transfected with the reporter construct pH100-TKluc and various DNAs as indicated. Cells were harvested 48 h post-transfection and assayed for luciferase activity. F.O.B, Fold Over Basal. C, co-precipitation of coactivators with clock proteins in HeLa cells. Lysates from quiescent HeLa cells was split in equal parts and immunoprecipitated (IP) with antibodies against Clock and Npas2 and rabbit and guinea pig IgG as controls. Immunocomplexes were probed for p300, CBP, and PCAF. D, Npas2 displays a time-dependent association with p300 in serum-induced HeLa cells. Representative blot of a single time course experiment (panel I). Representative expression of input p300 and β-actin in serum induced cells (panel II). Quantitative analysis of p300:Npas2 interaction (panel III). p300 bands were quantified using densitometry methods and normalized using β-actin values. Data are mean values ± S.E., n = 3.
INHAT (31), can all repress basal E-box activation of NPAS2:BMAL1 (Fig. 3D), providing further evidence for underlying HAT-dependent regulation of these circadian transcriptional co-regulators.

Analyses of Chromatin Remodeling and Coactivator Recruitment in Vivo—Real time PCR was used to examine mRNA expression profiles of Npas2, Bmal1, Clock, Per (1–2), Cry1, and Rev-erbα within cardiac tissue. This was obtained from Balb/c mice, which had been maintained in constant darkness. Hearts were harvested at 4-h intervals, over a 48-h period (Fig. 4A). Npas2 and Bmal1 cycled in the same phase, peaking at approximately ct1 and again at approximately ct29 (Fig. 4A, panel I). Clock expression does not cycle robustly throughout the daily period (Fig. 4A, panel I), as previously reported (9, 18). In addition, Rev-erbα mRNA oscillation was antiphasic to that of Bmal1 mRNA (Fig. 4A, panel II). This is in agreement with the finding that REV-ERBα can repress Bmal1 expression through binding to the ROR response elements in the promoter of Bmal1 (16). Both Per1 and Per2 cycled rhythmically and in phase with each other, but antiphase to the genes, which drive their oscillatory expression, Bmal1, Npas2, and Clock (Fig. 4A, panel III).

ChIP assays were performed on whole mouse heart homogenates at ct 6, 18, and 32 and analyzed using primers that bracket the most proximal E-box to exon 1 in the mPer1 promoter (Fig. 4B). Consistent with previous observations (27), time-dependent histone H3 acetylation, was observed on the proximal E-box, preceding the Per1 rhythm (Fig. 4A, panel III). This is in agreement with the in vitro data from serum induced HeLa cells, where the peak in histone H3 acetylation at T42 preceded Per1 maximum expression at T48 (Fig. 1B).

Immunoprecipitations using anti-rabbit IgG was used to determine nonspecific interactions of NPAS2 in whole mouse heart homogenates (Fig. 4C, panel I). Anti-NPAS2 immunoprecipitates showed significant temporal changes in both association and dissociation of p300 bound to NPAS2 (Fig. 4C, panel II). Interestingly, association was increased at T6 and T32 correlating with the rise of Per1 mRNA and the increase in histone H3 acetylation levels on the E-box surrounding its promoter (Fig. 4A, panel III and Fig. 4B). In addition to regulating Per1, CLOCK and BMAL1 have been shown also to drive rhythmically Cry expression (10, 15, 39). The observation that NPAS2-p300 association also increases at T20, preceding peak Cry expression (Fig. 4A, panel II), is consistent with a pervasive role for HAT-dependent mechanisms in regulating clock gene expression. Finally, using E-box reporter transactivation studies, we observe CRY2 mediated repression on basal NPAS2 and CLOCK-mediated luciferase reporter gene activation (Fig. 4D). Importantly, overexpression of CBP was partially able to relieve this repression, indicating that modulation of histone acetylation may be the target of CRY mediated E-box repression (Fig. 4D).

**DISCUSSION**

Acetylation of core histones on target promoters has been closely linked to transcriptional gene activation (22). In the present study, the coincidence of a rhythm in histone H3 and histone H4 acetylation on the proximal E-box of hPer1 with transcriptional activation of per1 and per2 is consistent with the heterodimeric complexes of CLOCK, NPAS2 and BMAL1 recruiting a histone acetyltransferase (HAT)-containing transcriptional co-activation complex to achieve maximal target gene activation. We considered p300, CBP, PCAF, and ACTR as potential circadian coactivators, all of which are known to possess acetyltransferase activity (40–42). Other known members of the bHLH-PAS family, such as HIF-1α and the AHR: AHRNT heterodimer, utilize p300/CBP to augment gene transcription (35, 36).
We have demonstrated an interaction between both CLOCK and NPAS2 with p300, CBP, PCAF, and ACTR by in vitro interaction assays. Importantly, this interaction does not constrain the enzymatic activity of p300 and PCAF, allowing them freely to acetylate core histones. The interactions of CLOCK and NPAS2 with p300, CBP, and PCAF were also observed in resting cells. Indeed, the binding of CBP to CLOCK and of PCAF to NPAS2, raises the possibility of selective association of proteins to elements of the clock transcription complex in a manner which might relate to tissue type and/or activation stimulus. Thus, p300 interacts in a time-dependent manner with NPAS2 under synchronized conditions. Increased association was observed at the time of maximal RNA expression, suggesting that temporal coactivator recruitment is a necessary step in driving circadian rhythmicity. This association is dramatically reduced at T12 and T36 corresponding with Cry2-mediated repression of Per1 and Per2. This finding agrees with the model proposed by Etchegaray et al. (27) that Cry proteins may disrupt the Clock associated transcriptional coactivator complex.

Cell-based luciferase assays using an E-box reporter revealed that the transcriptional activity of CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers is enhanced in the presence of CBP and PCAF and a further increase in activity occurs when CBP and PCAF are co-expressed together. CBP and PCAF are known to act cooperatively to enhance transcriptional activity of both FKLF2 (43) and IFN-β/H9252, the latter one of the best characterized transcriptional switches in eukaryotic cells (44). In the present studies, basal E-box activation was sensitive to inhibition by enzymes and complexes, which are known to inhibit or block HAT activity or function, highlighting the importance of HAT activity in regulating circadian gene expression.

A time-dependent recruitment of chromatin remodeling machinery by NPAS2 was also observed in vivo. Increased association of NPAS2 with p300 resulted in marked acetylation of N-terminal lysine residues on histone H3 surrounding the proximal E-box on the Per1 promoter in mouse heart homogenates, which was synchronous with circadian variability in its mRNA levels. The mechanism by which the cryptochrome proteins potently inhibit CLOCK/NPAS2:BMAL1-mediated transcription is presently unclear (15). However, overexpression of CBP was partially able to relieve this repression, indicating that modulation of histone acetylation may be the target of CRY-mediated E-box repression (Fig. 4D).
Targeted histone acetylation surrounds the proximal E-boxes on the Per1 promoter, causing localized chromatin remodeling and enhanced assembly of the basal transcription machinery. This regulates circadian gene expression in the cardiovascular system. Periodic recruitment of p300 to NPAS2 facilitates time-dependent chromatin remodeling and CRY2-mediated repression is induced by disruption of HAT-associated complexes on core clock heterodimers. These studies provide insight into the regulation of circadian rhythms in the cardiovascular system and elucidate further the fundamental role of chromatin remodeling in mammalian circadian biology.

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