Mass spectrometry-based absolute quantification reveals rhythmic variation of mouse circadian clock proteins

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Absolute values of protein expression levels in cells are crucial information for understanding cellular biological systems. Precise quantification of proteins can be achieved by liquid chromatography (LC)–mass spectrometry (MS) analysis of enzymatic digests of proteins in the presence of isotope-labeled internal standards. Thus, development of a simple and easy way for the preparation of internal standards is advantageous for the analyses of multiple target proteins, which will allow systems-level studies. Here we describe a method, termed MS-based Quantification By isotope-labeled Cell-free products (MS-QBiC), which provides the simple and high-throughput preparation of internal standards by using a reconstituted cell-free protein synthesis system, and thereby facilitates both multiplexed and sensitive quantification of absolute amounts of target proteins. This method was applied to a systems-level dynamic analysis of mammalian circadian clock proteins, which consist of transcription factors and protein kinases that govern central and peripheral circadian clocks in mammals. Sixteen proteins from 20 selected circadian clock proteins were successfully quantified from mouse liver over a 24-h time series, and 14 proteins had circadian variations. Quantified values were applied to detect internal body time using a previously developed molecular timetable method. The analyses showed that single time-point data from wild-type mice can predict the endogenous state of the circadian clock, whereas data from clock mutant mice are not applicable because of the disappearance of circadian variation.

absolute quantification | mass spectrometry | cell-free protein synthesis system | mammalian circadian clock protein | targeted proteomics

Quantitative information on protein expression levels is important to define the dynamic state of cells. Accurate measurements of absolute protein abundance in cells can be performed by emerging quantitative proteomics approaches such as selected reaction monitoring (SRM) or high-resolution mass spectrometry (MS) in combination with isotope dilution strategies (1). The methods require a known concentration of internal standards, typically prepared as the tryptic digests of target proteins, which are labeled with isotopically heavy atoms. The standard peptides are combined with samples containing the same peptides, and the mixtures are analyzed by MS. Quantities of peptides, which represent the target proteins, can be calculated by comparing ion intensities for isotopically light and heavy peptides.

The preparation of isotope-labeled peptides plays a major role in these approaches. The most common way to prepare such peptides is by absolute quantification, which uses chemical synthesis of peptides containing isotopically labeled amino acids (1, 2). It provides not only normal proteotypic peptides, but also modified peptides that mimic posttranslational modification. However, the method has several limitations originating from the use of the chemical synthesis. It requires individual peptide synthesis using large amounts of isotopically labeled amino acids as substrates. Difficulties of synthesis vary depending on the peptide sequence. Due to these features, it is difficult to reduce the cost per peptide, which leads to difficulties in preparing large numbers of peptides for the quantification. Multiple peptide production enables not only the multiplexed protein quantification but also highly sensitive detection and quantification of the proteins of interest by selecting effective internal standards with good signal/noise ratio from candidate peptides (3).

Several methods using a cellular gene-expression system have been developed in recent years for multiple peptide production. Multiple peptides can be obtained by the proteolysis of recombinantly expressed full-length proteins of interest (4, 5) or artificial proteins that compose a set of proteotypic peptides concatenated with each other (6, 7). These peptides are metabolically labeled with stable isotopes through *Escherichia coli* or other cellular gene-expression systems, allowing them to function as internal standards. The methods have advantages in that multiple peptides are produced from a single construct, which can be applied to both multiplexed and sensitive quantification.

A cell-free protein synthesis system is a useful tool for the expression of such proteins (3, 5, 6, 8). The cost for expensive stable isotope-labeled amino acids can be reduced because the volume for reaction mixtures is much lower than for culturing media. Protein expression and purification occurs in a high-throughput manner

Significance

A method for absolute quantification of proteins for targeted proteomics is developed. It introduces a simple and high-throughput synthesis of internal standards for peptide quantification and thereby facilitates both multiplexed and sensitive absolute quantification of proteins. Application of this method to the systems-level dynamic analysis of core circadian clock proteins and detection of internal body time using quantified values of circadian clock proteins is shown. The results demonstrate the validity of the developed method in which quantified values from wild-type mice can predict the endogenous state of the circadian clock.

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because there is no need for culturing, harvesting, and disrupting cells. Notably, cellular metabolism causes isotope scrambling and dilution, which is a problem where the homogeneity of isotope-labeled peptides is reduced due to conversion of labeled amino acids into others, or vice versa (9). This problem can be overcome in a cell-free system by artificial adjustment of the system components (10).

Here we describe a workflow for multiplexed absolute quantification of proteins using SRM-based targeted proteomics. The workflow, termed MS-based Quantification by isotope-labeled Cell-free products (MS-QBiC), has several features that expand advantages of the internal standard synthesis using a cell-free system. It is based on the use of the PURE system, a reconstituted cell-free protein synthesis system (11). Because the PURE system consists of purified factors and enzymes for *E. coli* translation machinery, synthesized peptides are rarely challenged by protease degradation that usually occurs in cell-extract systems. Additionally, isotope scrambling or dilution is avoided without the need to adjust system components (10). The developed workflow was applied to the absolute quantification of core circadian clock proteins in mouse livers across the circadian day. To obtain optimal peptides for the detection and quantification by the SRM-based targeted proteomics analysis, we synthesized 120 peptides for 20 circadian clock proteins. All of the peptides were successfully synthesized from PCR-amplified genes by the PURE system. Dynamic changes of copy numbers for 16 proteins during the circadian day were successfully quantified, demonstrating the potential of this method for the multiplexed targeted proteomics approaches.

Results

Design of the MS-QBiC Workflow. To develop a simple strategy for the multiplexed absolute quantification of protein using SRM-based targeted proteomics, we devised the MS-QBiC workflow (Fig. 1). This method takes advantage of the PURE system, a reconstituted cell-free protein synthesis system (11) for internal peptide synthesis without peptide degradation and without isotope scrambling or dilution (10). It is also noteworthy that the PURE system is suitable for protein or peptide expression from linear DNA, which enables the direct addition of the PCR-amplified gene into reaction mixtures. Thus, gene preparation, peptide synthesis, and purification can be performed in a simple and a high-throughput manner. The whole process can be carried out in 1 d from one or two DNA primers per peptide.

After examination and validation of the workflow by several liquid chromatography (LC)-MS analyses (Fig. S1 and *SI Results*), the MS-QBiC peptide was designed as shown in Fig. S2. A FLAG tag was selected as a purification tag and the quantification tag was obtained from the tryptic digest of bovine serum albumin (BSA), which was attached downstream of the FLAG tag and a spacer peptide sequence. The gene for the peptide was cloned into the plasmid vector comprising a T7 promoter and the 5′-UTR sequence for the *E. coli* translation system. The target peptide was designed to be directly attached to the quantification tag by PCR using the plasmid as a template. The MS-QBiC peptide can be quantified by comparing ion peak intensities of the stable isotope-labeled quantification tag with those of the chemically synthesized quantification tag (Fig. S2, *Lower Left*) or the tryptic digest of commercially available BSA (Fig. S1*D*). The quantified MS-QBiC peptide can be then used to quantify the target peptide by comparing the intensities of stable isotope-labeled target peptide in MS-QBiC peptide with those of the nonlabeled target peptide in the sample (Fig. S2, *Lower Right*).

The workflow was further examined for SRM analysis. Both the quantification of the MS-QBiC peptide and the data collection for the development of the SRM method for the target peptide (Fig. S3 *A*–*C*) were carried out by the LC-SRM analysis using mixtures containing digested MS-QBiC peptide and the chemically synthesized quantification tag. The developed SRM method was then applied to target peptide quantification in mouse liver.

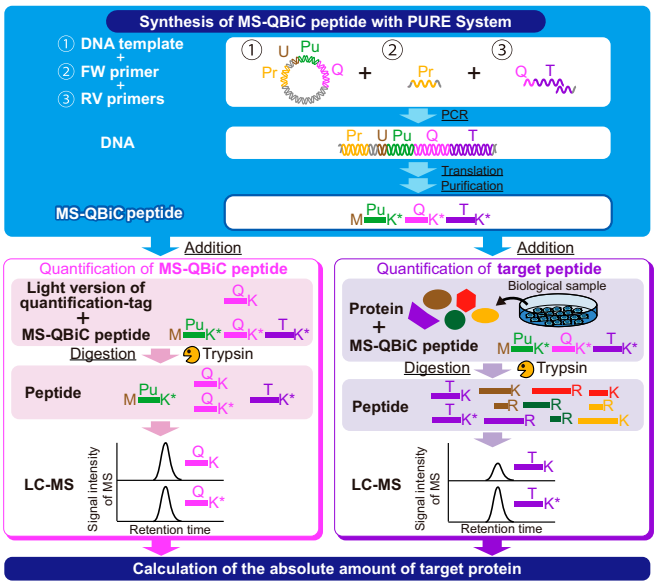

Fig. 1. Development of the MS-QBiC workflow. Schematic description of the MS-QBiC workflow. A purification tag, a quantification tag, and a tryptic peptide of the target protein (target peptide) are sequentially arrayed as a single peptide sequence (MS-QBiC peptide). The target peptide sequence is attached by one- or two-step PCR. The MS-QBiC peptide is synthesized in the PURE system in the presence of stable isotope-labeled Arg and Lys for isotopic labeling both the quantification tag and the target peptide. Trypsin digestion of purified MS-QBiC peptide produces equal amounts of isotopically labeled quantification tag and target peptide. The quantification tag is used to measure purified MS-QBiC peptide, and the target peptide is used as an internal standard for the target protein quantification.

The quantified MS-QBiC peptide was mixed with cellular protein extracts from a mouse liver and subjected to tryptic digestion and LC-SRM analysis. By comparing the ion peak intensities of the fragment peptides, the quantification of the target peptide could be performed (Fig. S3*D*), indicating that the MS-QBiC workflow can be applied to the SRM analysis.

Quantification of Core Circadian Clock Proteins. The workflow was applied to the quantification of core circadian clock proteins. The mammalian circadian clock is driven through complex feedback and feed-forward loops regulated by a variety of circadian clock proteins and three clock-controlled elements (12–15) (Fig. 2*A*). Although quantitative analyses of mRNA-level gene-expression of circadian clock proteins have been performed previously (13, 16), direct quantification of these proteins across the circadian day is essential for the accurate description and simulation of circadian clock networks. According to previous mRNA-level gene-expression profiles (13, 16), 20 circadian clock proteins were examined and 120 tryptic peptides were selected as target peptides for quantification of these proteins (Fig. 2 *A* and *B*, Datasets S1 and S2, and *SI Results*). Note that some peptides overlapped a common region of similar genes for DEC1 (BHLHE40) and DEC2 (BHLHE41), CKIδ and CKIε, REV-ERBα (NR1D1) and REV-ERBβ (NR1D2), RORα and RORβ, and DBP and TEF.

An experimental workflow used for quantification of circadian clock proteins is shown (Fig. 2*C*). Mice were entrained in a 12-h light/12-h dark (LD) cycle for 2 wk. After transferring to constant darkness, mice livers were collected at six time points with 4-h intervals at circadian times (CT0, CT4, CT8, CT12, CT16, and CT20). Livers from two mice were collected at each time point. The 120 synthesized and purified MS-QBiC peptides were mixed with protein extracts from the mice liver. The mixtures were subjected to tryptic digestion and then prefractionated with super

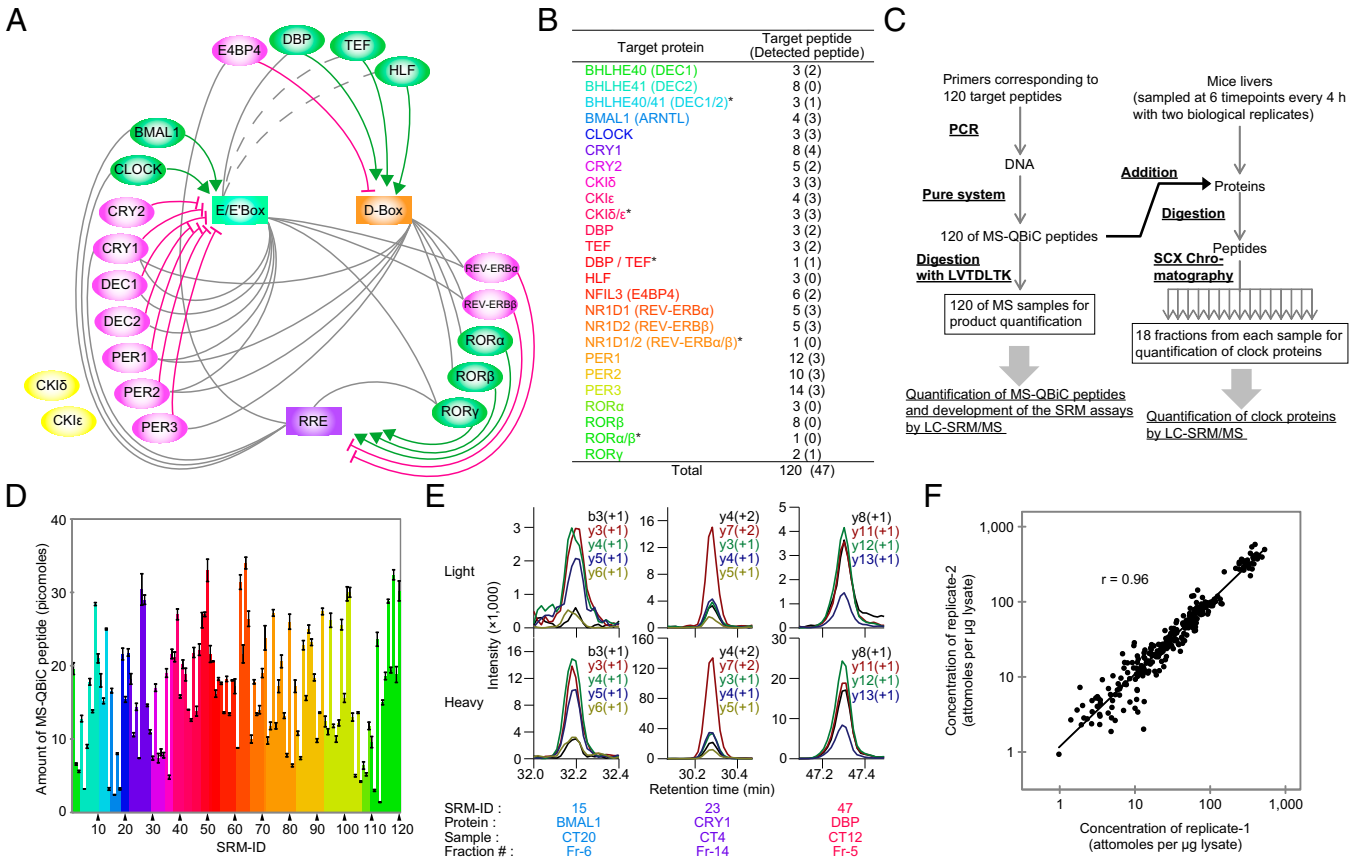

Fig. 2. Quantification of 20 circadian clock proteins in mice livers. (*A*) The transcriptional circuit of the mouse circadian clock. Clock-controlled elements (CCE; rectangles), transcriptional factors (green or magenta ovals) with their effects (lines), and kinases (yellow ovals) are shown in the circuit. Green ovals represent activators, and magenta ovals represent repressors. Gray lines indicate that the CCE is located on the promoter (or enhancer) regions of the corresponding transcription factor genes. Dashed lines indicate that the putative CCE is encoded. (*B*) A list of target proteins to be quantified. The numbers indicate the number of prepared target peptides and, in parentheses, the number of detected target peptides in the LC-SRM analyses. An asterisk indicates that target peptides are shared between two target proteins. (*C*) An experimental workflow for the absolute quantification of 20 circadian clock proteins. (*D*) Yields of 120 MS-QBiC peptides synthesized in the PURE system. The colors of each bar are same as those used in *B*. (*E*) Examples of SRM chromatograms corresponding to target peptides derived from clock proteins (*Upper*) and their internal standards (*Lower*). (*F*) Comparison of the quantified values between two biological replicates. Correlation coefficient (r) is indicated in the plot.

cation exchange (SCX) chromatography. The resultant 18 fractions were individually subjected to LC-SRM analysis (Fig. S4 and Dataset S2), and quantification of each peptide was performed. Prefractionation by SCX was adopted in the workflow because it was essential for the detection of proteins with low copy numbers (17, 18).

Quantification of the quantification tag showed that all of 120 MS-QBiC peptides were successfully synthesized from PCR-amplified genes by the PURE system. The amounts of purified peptide from the same volume of reaction mixtures (25 μL) ranged between 1.4 and 33.3 pmol (Fig. 2*D*). Although the yields were variable, implying dependency on the sequence of the target peptide, they were sufficient for our SRM-based quantification. These data demonstrate the versatility of our workflow for many peptides regardless of their sequence.

Quantification of the target peptide (Fig. 2*E*) showed that 47 peptides (42 peptides were derived from a single protein and 5 peptides were in the common region of two proteins) could be used for detection of circadian clock proteins in mice liver samples (Fig. 2*B* and Dataset S2). The remaining 73 peptides were classified into two classes: in one class, internal standards were not detected probably because of unsuccessful separation with SCX chromatography (black rows in Fig. S4*C*) and in the other class signal/noise ratios were not high enough for detection of endogenous targets. The quantified values of 47 peptides at six time points were compared between two biological replicates by a double-logarithmic plot (Fig. 2*F*). The quantified values were highly correlated between replicates, indicating the biological reproducibility of circadian clock protein dynamics as well as the technical reproducibility of the developed workflow. Fifteen proteins were detected with two or more internal standards whereas one protein, RORγ, was detected with a single internal standard (Fig. 2*A* and Fig. S5*A*). Peptides for the common region of DEC1 (BHLHE40) and DEC2 (BHLHE41), CKIδ and CKIε, and DBP and TEF were also successfully quantified, but four proteins [DEC2 (BHLHE41), HLF, RORα, and RORβ] were not detected clearly in our analysis (Fig. S5 *D* and *E* and *SI Results*).

Time courses for the concentrations of 16 proteins (Fig. 3*A*) were shown as averaged peptide concentrations derived from same protein with errors between peptides. The differences in concentration between peptides (Fig. S5*A* and Dataset S3) were relatively higher than those between biological replicates (Fig. S5*B* and Dataset S3). Various factors, such as posttranslational modification, the presence of unknown protein isoforms, and inefficient or missed cleavages by the proteolysis, in addition to measurement error itself, may cause these differences, which are common to MS-based absolute quantification (20). Despite these problems, the data showed similar values from peptides derived from same

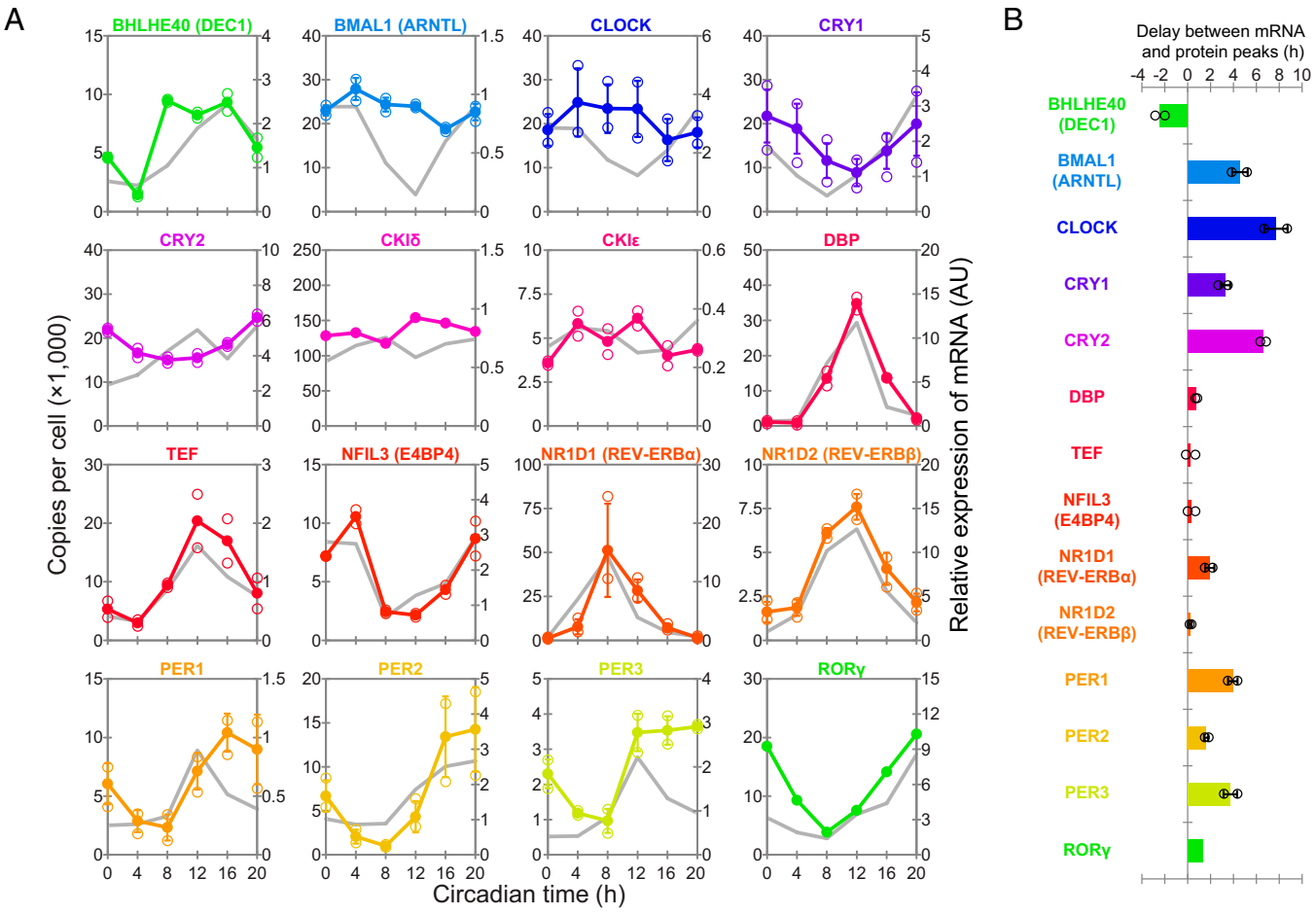

Fig. 3. A summary of the expression levels of circadian clock proteins and their corresponding mRNA. (*A*) Time course of the quantified proteins with their corresponding mRNA. Expression levels of the protein are shown in the same color as used in Fig. 2B. Concentrations are expressed as copies per cell (left axis). Copy numbers were calculated by assuming that the total amount of protein per cell is 0.5 ng (19). Bars on the graph indicate the SD of quantified values using three or more internal standard peptides. Open circles indicate maximum and minimum quantified values using two or more peptides. Relative expressions of mRNA, which are the replots of Fig. S7, are shown in gray (right axis). (*B*) Phase delay of the variations between circadian clock proteins and their corresponding mRNA. The phases of each variation were calculated by 24-h cosine curve fitting to time-course data of each internal standard peptide and mRNA expression (Dataset S3). The bars indicate the SD of calculated phases of proteins using three or more internal standard peptides. Open circles indicate the maximum and minimum phase delay calculated from the phases of proteins using two or more internal standard peptides.

protein, which suggests that the observed values reflect absolute copy numbers in cells. Six proteins [DEC1 (BHLHE40), CRY2, DBP, CKIε, TEF, and NFIL3 (E4BP4)] were quantified with only two target peptides, and two proteins (CKIδ and RORγ) were quantified with only a single peptide. Although target peptides were selected from as many as possible according to our criteria (Dataset S2 and *SI Results*), the number of detected peptides depends on both a trypsin cleavage site in the amino acid sequence of the protein and the signal/noise ratio of each target peptide in the SRM analysis. The use of alternative proteases may increase the number of usable peptides for more reliable quantification. However, absolute quantification depends on the efficiency and specificity of the selected proteases, which is highest with the use of a tandem LysC (Lysyl Endopeptidase)/trypsin cleavage (Fig. S6). Thus, this method is currently the best way for MS-based protein quantification (21) and our studies (*SI Results*). To show errors between peptides for the six proteins, maximum and minimum quantified values from each measurement were additionally plotted in the graph (Fig. 3*A*). It should be noted that peptide-1 and peptide-3 from CKIδ and peptide-1 from CKIε (Fig. S5*A*) were excluded from protein quantification. The concentrations of these peptides were consistently lower compared with the maximum peptide concentration for each protein, i.e., the coefficient of variation between peptides was more than 50% at all time points (Dataset S3). Additional experiments showed that these peptides were phosphorylated (Fig. S5*C*), which indicated that the peptides were not suitable for quantification. Phosphorylation of peptide-1 from CKIε was also reported previously in phosphoproteomics analyses including that of mice liver (22, 23).

The relative abundance of the mRNA corresponding to each protein, obtained by quantitative PCR (qPCR) using livers from same samples (Fig. S7), is additionally shown in the time course in Fig. 3*A*, which shows that copy number of the 14 proteins, as well as the abundance of mRNA, fluctuated across the circadian day except for CKIδ and CKIε (Dataset S3). The phase differences between the proteins and mRNA, calculated by a 24-h cosine curve fitting to the time-course data, varied with each protein (Fig. 3*B*). There was relatively little phase delay for some proteins [DEC1 (BHLHE40), DBP, TEF, E4BP4 (NFIL3), REV-ERBα (NR1D1), REV-ERBβ (NR1D2), PER2, and RORγ], indicating that these protein levels are governed mainly by mRNA abundance. Phase differences were relatively large for BMAL1, CLOCK, CRY1, CRY2, PER1, and PER3, suggesting that these expression levels are regulated through complicated processes, including

posttranscriptional regulation, translation control, posttranslational modification, and degradation (24–28). These observations demonstrated that the direct quantification of proteins is essential for the description of protein-level dynamics of biological systems.

Circadian variation of PER1, PER2, CRY1, and CRY2 was investigated previously by Western blot analyses (24). The time points for the maximum and minimum quantity of these proteins in that report corresponded well with this study. Both studies showed that the amplitudes of PER1 and PER2 were larger compared with CRY1 and CRY2. In contrast, significantly high variations of BMAL1 and CLOCK expression level observed in the Western blot analysis were not observed in our results. Although we have no explanation for this inconsistency, our observations suggested that the amplitude of the variation of these proteins was relatively low. It is noteworthy that the copy numbers and variations of these proteins matched each other, which reflects that they function through the formation of a heterodimer (29). It is interesting that these copy numbers also matched CRY1 at CT0, the peak time for CRY1. A recent report suggested that the three proteins form a ternary complex to inhibit the E/E′-Box promoters at this time point (30). Finally, the mRNA-level expression of all of the proteins, except for CKIδ and CKIε, is known to fluctuate across the circadian day (13), which is consistent with our analysis.

Construction of a Molecular Timetable. The quantified values of peptides for 14 circadian clock proteins except for CKIδ and CKIε showed circadian rhythmicity (Dataset S3). The time points for maximum or minimum quantity varied among the proteins. Our previous studies demonstrated that such data sets can provide a molecular timetable that acts as a reference for body-time (BT) detection from only one or two time points (31–33). BT detection has been performed for mRNA-level gene-expression profiles of mouse cells and the mouse and human blood metabolite profiles. In the present study, the method was further applied for protein-level expression profiles using the quantified values of each peptide to show whether they indicate the endogenous state of the circadian clock.

To construct a molecular timetable, the quantified values of 36 peptides for 14 circadian clock proteins at each time point were normalized according to the previous study. Peak time of each peptide was calculated by the time-course data of the normalized values (Fig. 4*A* and Dataset S3), which indicated the molecular timetable of each peptide. The calculated peak time corresponded well if the peptide originated from a same protein, suggesting that the molecular timetable of each peptide represented that of the corresponding protein. The normalized quantity of each peptide at each time point was plotted against the calculated molecular timetable, and then 24-h period cosine curves were fitted to these plots, where the peak time of the best-fitted curve indicated the estimated BT (Fig. 4*B*). The estimated BT matched well with the actual circadian phase with a mean error of 0.67 ± 0.29 h, demonstrating that a molecular timetable can be constructed for the protein-level expression profiles.

BT Detection in Wild-Type and Clock Mutant Mice. To validate the molecular timetable based on the protein-level expression profiles, mice livers were further collected at CT4 and CT16 with two biological replicates. The quantified values of 36 peptides from livers were applied to BT estimation using the molecular timetable method (Fig. S8*A*). The livers at CT4 were estimated to be 3.7 and 3.3 h and those at CT16 to be 17 and 16.5 h, respectively, which were close to the actual circadian phase.

The circadian clock proteins in clock mutant mice were additionally quantified using the MS-QBiC method. Livers from three knockout (KO) or double knockout (DKO) mice (*Bmal1* KO, *Per1/2* DKO, and *Cry1/2* DKO), which were previously shown to have deficiency in the circadian rhythmicity (34–36), were collected at CT4 and CT16, respectively, with three biological replicates. Quantified values of 16 proteins based on the quantification of 47 peptides showed a markedly different behavior compared with those of the wild-type mice (Fig. S9 and *SI Results*). The large differences of the expression level of DEC1 (BHLHE40), DBP, TEF, E4BP4 (NFIL3), PER1, PER2, and PER3 between CT4 and CT16 in wild-type mice were diminished in the mutant mice, consistent with the deficiency in circadian rhythmicity.

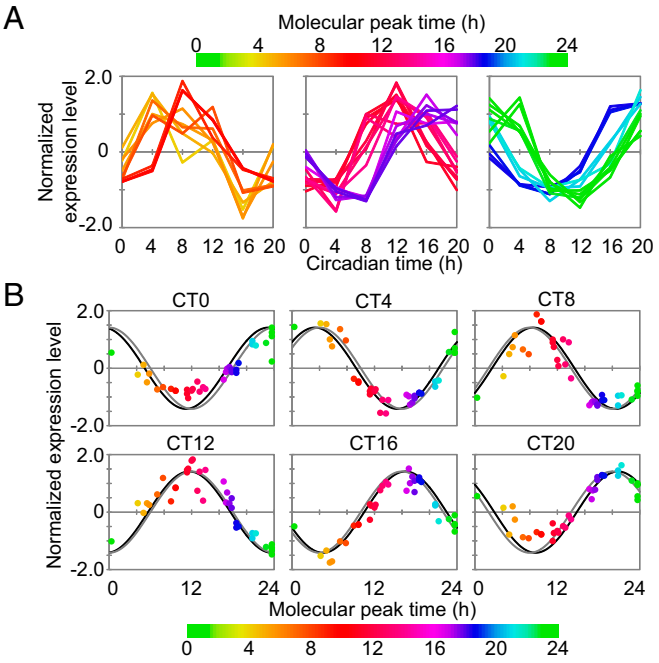

Fig. 4. Molecular timetable based on the protein expression of core clock genes. (*A*) Time course of normalized expression levels of peptides. The colors—in ascending order from green to orange (CT2-10, *Left*), from orange to purple (CT10-16, *Center*), and from purple to green (CT16-2, *Right*)—were assigned to represent the molecular peak time, which corresponds to the molecular timetable of each peptide. The color code is represented above the diagrams. (*B*) Expression profiles of peptides at each circadian time point. Normalized expression levels of peptides at each circadian time point are plotted against their molecular peak time. Best-fitted cosine curves (black) and those based on the actual circadian time point (gray) are shown. The peak time of the best-fitted curves indicates the estimated BT.

The quantified values of 36 peptides for 14 circadian clock proteins were further applied to BT estimation of clock mutant mice using the molecular timetable of the wild-type mice as a reference. The plots of the normalized quantity of each peptide against the molecular timetable were similar between CT4 and CT16 in each sample and indicated the same estimated time, which reflects a deficiency in circadian rhythmicity (Figs. S8 *B–D* and S10). Considering that the variation of the expression of circadian clock proteins was diminished in the mutant mice (Fig. S9), similar profiles between CT4 and CT16 may suggest that the expression level of these proteins is constant across the circadian day (Fig. S8*E*).

Discussion

As the technologies for MS-based proteomics advance, quantification of entire cellular protein networks has become feasible (37–39). However, it is still a difficult task to quantitatively investigate dynamics of the networks using global proteomics approaches by comparatively analyzing multiple cellular extracts under a variety of environments or perturbations. The quantification becomes far more difficult for low abundant proteins, such as transcription factors and kinases. Two recent global proteomics studies have revealed various proteins with circadian rhythmicity

by analyzing time-series samples from large numbers of mice livers (40, 41). However, core circadian clock proteins, which consist mainly of transcription factors and kinases, were not detected.

Targeted proteomics has grown in recent years as a strategy to compensate for the weaknesses of global proteomics approaches. It monitors only signal intensities with good signal/noise ratios derived from target peptides under optimal conditions. It can analyze low-abundance proteins more effectively, but the number of proteins that can be analyzed is reduced compared with the global proteomics approaches. Therefore, targeted proteomics approaches are suitable for analyzing the dynamics of specified biological systems comprising low-abundance proteins.

In this study, we performed systems-level analysis of dynamics of core circadian clock proteins by using SRM-based targeted proteomics. Sixteen proteins from 20 circadian clock proteins were successfully quantified using time-series samples from mice liver, and 14 proteins had circadian variation (Fig. 3 and Dataset S3). The developed method can be applied to other organs or tissues such as the brain that function as a central circadian clock. It will be interesting to quantify the core circadian clock proteins in the brain to compare the abundance between the central and peripheral organs using the developed method for future studies.

Four proteins [DEC2 (BHLHE41), HLF, RORα, and RORβ] were not clearly quantified, although estimation of two proteins (HLF and RORα) was examined from peak intensities with low signal/noise ratios (Fig. S5*E*). The quantification result from the common region of DEC1 (BHLHE40) and DEC2 (BHLHE41) suggests that the expression level of DEC2 is extremely low (Fig. S5*D*), and this may have resulted in unsuccessful detection of the signal peaks for DEC2. The detection limit of DEC2 (BHLHE41) and RORβ, estimated as threefold of the SD of noise peaks, is about 1,000 and 100 copies per cell, respectively, when the most sensitive standard peptides are used (Dataset S3). Thus, further development of the SRM-based detection as well as the sensitivity of MS itself is necessary for the detection of proteins with extremely low expression levels (<1,000 copies per cell).

The MS-QBiC workflow allows for multiplexed preparation of isotopically labeled peptides for use as internal standards. Multiple peptides can be prepared in parallel without any cDNA cloning or gene synthesis step by using the MS-QBiC workflow. The use of the reconstituted cell-free protein synthesis system avoids isotope scrambling and dilution. Moreover, multiplexed peptide preparation can increase the sensitivity of SRM analysis by developing an optimal method that depends on the physical properties of individual peptides. Although the yield of synthesized peptides differs from one peptide to another, sufficient amounts of the peptide were successfully prepared regardless of their sequences, suggesting versatility in the developed workflow. This versatility also suggests that the developed method does not rely on the characteristics of proteins and can be applied to proteins that are difficult to prepare recombinantly, such as membrane-bound proteins and other DNA-binding proteins. Currently, SRM analyses highly depends on the performance of the MS, and thus detection of several hundreds of proteins is estimated to be the upper limit according to previous reports (39, 42). However, this limit can be increased by improvement of MS performance. Therefore, high-throughput synthesis of internal peptides using the PURE system can be adapted to such improvements in the future. Presently, the developed method is not able to be applied to the preparation of modified peptides that mimic posttranslational modification. However, integration of the developed method with technologies based on genetic code engineering (43), including site-specific incorporation of phosphoserine residues into the proteins or peptides (44), has the potential to overcome this difficulty, and we are now exploring this possibility.

The core circadian clock proteins consist mainly of transcription factors, which bind to DNA and function as activators or repressors for transcription. Interactions between DNA and BMAL1, CLOCK, CRY1, CRY2, PER1, and PER2, which play crucial roles in the formation of circadian rhythmicity, have been comprehensively analyzed using ChIP-seq analyses (45) demonstrating that binding of these proteins to their binding sites shows circadian rhythmicity. The peak time of binding matches well with that of the protein concentrations quantified in this study, except for CRY2, suggesting that the binding is principally regulated by the expression levels of each protein. However, the binding may be regulated not only by the quantity of protein, but also by other qualities (e.g., posttranslational modification status): The amplitude of BMAL1, CLOCK, CRY1, and CRY2 is quite low, and a considerable amount of the proteins may remain in cells at a circadian phase when their binding to DNA is low and the peak time of CRY2 concentration is different from that of its DNA binding. These observations suggest that qualitative changes of these proteins are also important for the DNA-binding activities. Calculating from our results and the ChIP-seq data, the numbers of proteins at each binding site are 5.6 BMAL1, 14.6 CLOCK, 7.2 PER1, 3.2 PER2, 3.4 CRY1, and 4.8 CRY2 at a single binding site, respectively. The values suggest that 3–15 proteins are necessary at a binding site to regulate gene expression for the formation of the circadian clock.

The application of the molecular timetable methods to the quantified peptides from 14 circadian clock proteins demonstrates that single-time-point data can predict body time, indicating that expression levels of each circadian protein are promising markers for future application of body time detection in human samples. The accuracy of detection is comparable to the mRNA-level expression-based timetable method rather than the metabolite-based method, which requires two-time-point data for detection (31–33). Furthermore, the constructed timetable, which is based on the absolute quantification of cellular proteins, can be universal compared with the previous two methods based on the relative quantification, which may enable the single-time-point assay in different places. Resemblance of peptide expression profiles between CT4 and CT16 in three mutant mice suggests that the expression level of the proteins is fixed at the level of the designated time point. In *Bmal1* KO mice, the absence of CLOCK/BMAL1 heterodimer represses the E/E′-Box promoters and then activates the RRE promoters. In *Per1/2* DKO and *Cry1/2* DKO mice, the absence of PER1 and PER2 or CRY1 and CRY2 activates the E/E′-Box promoters and in turn activates the D-Box promoters whereas the RRE promoters are suppressed to some extent (Figs. S8*E* and S9). Such fixation of the activities of each promoter may result in the stalled circadian gene-expression dynamics at the specific circadian phase.

Materials and Methods

Animals. All mice were carefully kept and handled according to the RIKEN Regulations for Animal Experiments. See *SI Materials and Methods* for details.

Sample Preparation. Chemically synthesized peptides, MS-QBiC peptides, cell/nuclear lysates from HEK293T cells, and mouse liver samples were prepared for this study. They were subjected to enzymatic digestion and prefractionation before MS analyses. See *SI Materials and Methods* for details.

MS Analyses. Two types of mass spectrometers—an Orbitrap mass spectrometer and a triple quadruple mass spectrometer—were used in this study. See *SI Materials and Methods* for details.

RNA Expression Analysis of Circadian Clock Genes by qPCR. The mRNA-level expression profiles of circadian clock genes were measured by qPCR. See *SI Materials and Methods* for details.

BT Detection by Molecular Timetable Methods. Estimation of body time by using expression profiles of circadian clock proteins was performed according to the molecular timetable methods as reported previously (31). See *SI Materials and Methods* for details.

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