Role of Phosphorylation in the Mammalian Circadian Clock

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Circadian clocks regulate a wide variety of processes ranging from gene expression to behavior. At the molecular level, circadian rhythms are thought to be produced by a set of clock genes and proteins interconnected to form transcriptional-translational feedback loops. Rhythmic gene expression was formerly regarded as the major drive for rhythms in clock protein abundance, but recent findings underline the crucial importance of posttranslational mechanisms for both the generation and dynamics of circadian rhythms. In particular, the reversible phosphorylation of PER proteins—essential components within the negative feedback loop in *Drosophila* and mammals—seems to have a key role for the correct timing of nuclear repression. To understand how PER protein phosphorylation regulates the dynamics of the circadian oscillator, we have mapped endogenous phosphorylation sites in mPER2. Detailed investigation of the functional role of one particular phosphorylation site (Ser-659, which is mutated in the familial advanced sleep phase syndrome [FASPS]) led us propose a model of functionally different phosphorylation sites in PER2. This concept explains not only the FASPS phenotype, but also the effect of the *tau* mutation in hamster.

INTRODUCTION

Most light-sensitive organisms have evolved internal clocks that regulate daily rhythms in physiology, metabolism, and behavior. In a natural environment, these clocks are synchronized to external zeitgebers, such as the light/dark cycle or temperature cycles, to ensure a stable phase relationship between internal and external processes. Under constant conditions, oscillations are free-running with a period close to 24 hours, hence the term *circa-dian* clocks. Extensive studies in the last two decades have unraveled the molecular basis of circadian rhythmicity in a broad variety of organisms such as cyanobacteria, plants, *Neurospora*, *Drosophila*, and mammals. Conceptually, circadian rhythms are generated by delayed negative feedback loops within single cells. A set of so-called clock genes and clock proteins are interconnected to produce self-sustained circadian oscillations at the molecular level.

In mammals, a negative gene-regulatory feedback loop is at the heart of the circadian oscillator: CLOCK and BMAL1—bHLH (basic helix-loop-helix)–PAS (Period-Arnt-Single-minded)-containing transcription factors—heterodimerize and bind to E-box enhancer elements in the promoter region of the *Period* (*Per1*, *Per2*) and *Cryptochrome* (*Cry1*, *Cry2*) genes to activate their transcription. PER and CRY proteins, together with casein kinase 1 ϵ/δ (CK1 ϵ/δ) and probably other proteins, form a large multimeric complex, the circadian feedback module (Brown et al. 2005a; Hofmann et al. 2006). It is believed that after some time, the complex enters the nucleus, where PER and CRY proteins inhibit their own synthesis by directly interacting with the CLOCK-BMAL1 heterodimer. At present, the exact composition of the module is unclear, although multiple additional proteins have been suggested to contribute to the periodic inhibition of CLOCK-BMAL1 activity. These include mammalian Timeless (Sangoram et al. 1998; Barnes et al. 2003), the

bHLH transcription factors DEC1/2 (Honma et al. 2002), the PER1-associated proteins NONO and WDR5 (Brown et al. 2005a) as well as the CLOCK-interacting protein CIPC (Zhao et al. 2007).

Further feedback loops possibly contribute to the robustness of the system. In a positive feedback loop, the transcription of the *Bmal1* gene is regulated by rhythmic action of Rev-Erb α , the transcription of which is also controlled by CLOCK-BMAL1 via E-box elements (Preitner et al. 2002).

Critical for the generation of a self-sustained circadian oscillation with a period of about 24 hours is a time delay of several hours between the synthesis of PER and CRY proteins and their action as inhibitors of their own expression in the nucleus. Several mechanisms are discussed to participate in the generation of this delay. These mechanisms modulate abundance, localization, and activity of transcriptional inhibitors by regulating transcription, translation, and posttranslational modifications of important components of the circadian oscillator.

Up to now, many posttranslational mechanisms have been discovered to modulate circadian dynamics, although we are far from having a comprehensive view on the function of posttranslational modifications for the clock. Perhaps the most important posttranslational modification in eukaryotic cells is the phosphorylation of serine, threonine, and tyrosine residues. Such phosphorylations frequently act as molecular switches between active and inactive protein states either directly by regulating the activity (e.g., kinases) or indirectly by altering subcellular localization (e.g., transcription factors). In addition, phosphorylation often initiates protein degradation by the proteasome. Target proteins contain specific amino acid motifs, which, when phosphorylated, form a recognition sequence for the ubiquitin ligase complex.

Several important components of the mammalian circadian oscillator are known to be targets of posttranslational modifications. CLOCK, BMAL1, PER, and

possibly also CRY proteins are phosphoproteins in vivo (Lee et al. 2001). Moreover, BMAL1 was shown to be sumoylated (Cardone et al. 2005) and, very recently, acetylated (Grimaldi et al., this volume). These modifications specifically alter the properties and functionality of the corresponding proteins by changing protein stability, subcellular localization, activity, or complex formation.

Hitherto, phosphorylation is the best-explored post-translational mechanism within essentially all circadian model systems. Recent results from cyanobacteria even challenge the dogma of rhythmic transcriptional-translational feedback loops as the fundamental principle of rhythm generation: A purely posttranslational mechanism based on rhythmic phosphorylation and dephosphorylation of the hexameric KaiC protein is sufficient to generate and sustain circadian rhythms at least in vitro (Nakajima et al. 2005; Tomita et al. 2005). In flies and mammals, there is also evidence for the particular importance of posttranslational mechanisms: Constitutive expression of *dPer* in arrhythmic *per⁰* flies rescues arrhythmic behavior and leads to rhythmic PER protein levels (Yang and Sehgal 2001). Similarly, constitutive expression of mammalian *Per* genes in synchronized cultured cells also results in rhythmic PER protein abundance (Fujimoto et al. 2006; Nishii et al. 2006). Consequently, these data suggest that rhythmic PER protein abundance in vivo might be generated by a combination of rhythmic transcription of *Per* genes and posttranslational events. At least in the case of flies, the posttranslational mechanism seems to be sufficient for behavioral circadian rhythmicity.

This chapter focuses on the functional role of clock protein phosphorylation for the generation and the dynamics of circadian oscillations with a special emphasis on mammalian and *Drosophila* PER proteins.

Kinases and Phosphatases

Doubletime (DBT)—the *Drosophila* homolog of mammalian CKI ϵ —was the first kinase identified to have an important functional role in the circadian system of *Drosophila*. Flies with mutations in the *dbt* gene have altered circadian periods at both the behavioral and molecular levels. In *dbt* mutant flies, the stability, phosphorylation pattern, and subcellular localization of dPER is modified (Kloss et al. 1998; Price et al. 1998). Subsequent studies showed an additional role for DBT in the phosphorylation of dCLOCK, thereby regulating its stability (Kim and Edery 2006; Yu et al. 2006). In addition, *Drosophila* casein kinase 2 (dCK2) was shown to phosphorylate dPER. This phosphorylation predominantly functions in the regulation of the subcellular localization of dPER by promoting its nuclear entry and accumulation, whereas its effects on dPER stability are rather weak (Lin et al. 2002, 2005; Akten et al. 2003). Moreover, Shaggy—the *Drosophila* homolog of mammalian glycogen synthase kinase 3 β (GSK3 β)—phosphorylates dTIM, the essential binding partner of dPER, thereby promoting the nuclear entry of the PER/TIM heterodimer (Martinek et al. 2001). The impact of Shaggy phosphorylation on TIM stability has not yet been eluci-

dated, although it has been reported that phosphorylated TIM species are more prone to light-induced proteasomal degradation (Zeng et al. 1996).

The discovery of DBT's importance in the *Drosophila* circadian system initiated extensive studies to elucidate a possible conserved function of the DBT homologous kinases of the CKI family in the mammalian clockwork. Indeed, it has been demonstrated that CKI ϵ and CKI δ are involved in the phosphorylation of the mammalian PER proteins (Keesler et al. 2000; Camacho et al. 2001; Akashi et al. 2002; Schlosser et al. 2005). Moreover, CKI ϵ is able to phosphorylate BMAL1 and CRY proteins in vitro (Eide et al. 2002).

Recent studies showed that GSK3 β also has a role in the mammalian circadian system, but interestingly, its targets do not seem to be conserved. Whereas Shaggy phosphorylates dTIM in *Drosophila*, substrates of mammalian GSK3 β are CRY2 (Harada et al. 2005), Rev-erb α (Yin et al. 2006) and PER2 (Iitaka et al. 2005). Here, GSK3 β -mediated phosphorylation regulates the stability of CRY2 and Rev-erb α as well as the subcellular localization of PER2.

It is still unclear whether additional kinases are involved in the generation of circadian rhythmicity in mammals. However, at least in the case of mPER2, it has been speculated that yet unknown kinases are involved in phosphorylation-dependent subcellular localization (Vanselow et al. 2006).

Phosphatases are the natural opponents of kinases: They ensure reversibility of phosphorylation-induced alterations in protein function. The role of phosphatases in the circadian clockwork is just emerging. So far, it has been demonstrated in *Drosophila* that PP2A dephosphorylates and thus stabilizes dPER (Sathyanarayanan et al. 2004). In mammals, PP1 was shown to dephosphorylate a mPER2 fragment in vitro. In addition, coexpression with a dominant-negative version of PP1 destabilizes this mPER2 fragment, indicating that dephosphorylation of PER proteins by PP1 may counteract CKI ϵ / δ -induced proteasomal degradation (Gallego et al. 2006a). Furthermore, PP5 has been reported to regulate the kinase activity of CKI ϵ by antagonizing its inhibitory autophosphorylation. Interestingly, the PP5-mediated activation of CKI ϵ is inhibited by cryptochrome proteins (Partch et al. 2006), which may provide an explanation for the stabilizing effect of CRYs on PER proteins.

PER Phosphorylation

In the 1990s, a delayed negative feedback loop was proposed as the fundamental principle of circadian rhythm generation in *Drosophila*. This was primarily based on the striking 4–6-hour lag between the accumulation profiles of *Drosophila Per* mRNA and protein (Hardin et al. 1990), leading to speculations that a pure transcriptional mechanism is not sufficient to create this delay. When analyzing the circadian accumulation patterns of dPER and mammalian PERs by western blotting, a substantial electrophoretic mobility shift of the PER bands occurred, in addition to a high-amplitude variation in protein abundance. This mobility shift has been shown

to be due to a gradual, circadian phase-dependent phosphorylation of the PER proteins (Edery et al. 1994; Lee et al. 2001). Interestingly, the timing and extent of the PER phosphorylation are modified in period-altering fly mutants (Edery et al. 1994), indicating that the phosphorylation status of the PER proteins reflects the progress in the circadian cycle.

Several properties of PER proteins turned out to be directly regulated by the degree of PER phosphorylation, such as stability, subcellular localization, and inhibitory activity (see below). Importantly, the phosphorylation of PER proteins in *Drosophila* and mammals is not mediated by a single kinase but is the result of a complex temporal and spatial interplay of several kinases and phosphatases.

Phosphorylation regulates stability. Already in one of the first studies on *Drosophila* PER protein, it has been noted that the dPER species with the highest degree of phosphorylation are present at times right before the drastic drop of protein abundance. This suggested a direct link between dPER phosphorylation and degradation (Edery et al. 1994). In *dbt^P* larvae (a *dbt* loss-of-function mutant), dPER is hypophosphorylated and accumulates to unusual high levels (Price et al. 1998), further indicating that phosphorylation destabilizes PER. Subsequently, it was shown that progressive phosphorylation of dPER triggers the binding of the F box/WD40-repeat protein Slimb. Slimb functions as a substrate-recognizing component of the ubiquitin ligase SCF complex and promotes the ubiquitination and subsequent proteasomal degradation of hyperphosphorylated PER (Grima et al. 2002; Ko et al. 2002). As *slimb* mutant flies are behaviorally arrhythmic, the time-specific degradation of PER proteins is an essential part of circadian rhythm generation and maintenance. It adds to the required time delay within the negative feedback loop by counteracting a premature cytoplasmic and nuclear accumulation of the inhibitory complex.

In a very similar manner, mammalian PER proteins are extensively phosphorylated by CKI ϵ and CKI δ , thereby reducing the stability of the proteins by targeting them for proteasomal degradation (Keesler et al. 2000; Camacho et al. 2001; Akashi et al. 2002). PER proteins are recognized by the Slimb homologs β -TrCP1/2 as parts of the SCF ubiquitin ligase complex. The interactions between β -TrCP1/2 and their substrates occur via specific recognition sequences within the substrate. For both mPER2 and mPER1, a β -TrCP1/2-binding site has been proposed (Eide et al. 2005; Shirogane et al. 2005). Down-regulation of both endogenous β -TrCP1 and β -TrCP2 in synchronized fibroblasts results in a substantial lengthening of the circadian period. Furthermore, the expression of β -TrCP1/2 interaction-deficient PER2 variants in synchronized fibroblasts leads to a dramatic stabilization of PER2 protein as well as to a disruption of circadian rhythms (Reischl 2007).

In mammals, PER proteins are not the only clock components, whose stability is controlled by the proteasomal pathway. Very recently, the F-box protein Fbx13 was shown to target CRY proteins for proteasomal degradation. Mice with mutations in Fbx13 display a lengthened circadian locomotor activity rhythm, an increased stabil-

ity of the CRY proteins, and a reduced expression of PER proteins (Busino et al. 2007; Godinho et al. 2007; Siepka et al. 2007). Given the relatively high CRY protein amounts as well as the rather small amplitude of CRY protein abundance rhythms, the molecular basis of Fbx13's essential role in the circadian clock is still, at least in part, obscure. For example, it is unclear whether phosphorylation of CRY proteins is required for recognition by Fbx13.

Phosphorylation regulates subcellular localization. Phosphorylation affects not only PER protein stability, but also its subcellular localization. For example, in *dbt* and *per* mutant flies, besides the accumulation profile, the nuclear import and nuclear clearance patterns of dPER are altered. Furthermore, Cyran et al. (2005) showed that phosphorylation by DBT retains PER in the cytoplasm, whereas hypophosphorylated dPER is able to enter the nucleus in *tim⁰¹*; *dbt^P* double-mutant larvae and *tim⁰¹*; *dbt^{arr}* double-mutant flies, respectively. Thus, proper dPER phosphorylation by DBT is crucial for correct timing of dPER nuclear entry and export. Besides DBT, the phosphorylation of CK2 has a clear impact on the subcellular localization of dPER. Mutations in the catalytic α or regulatory β subunit of CK2 lengthen the circadian period of flies and concomitantly decelerate the nuclear entry of the PER protein (Lin et al. 2002; Akten et al. 2003). A similar role for CK2 has also been established in the *Neurospora* and *Arabidopsis* clock (Sugano et al. 1999; Yang et al. 2002). Given this striking conservation in several phylogenetic kingdoms, an important function for CK2 has been proposed in the mammalian system, but not yet established.

In mammals, the influence of CKI ϵ / δ phosphorylation on the subcellular localization of PER proteins is still not fully understood. Several independent studies have addressed this issue with ambiguous and sometimes contradictory results. In cultured cells, the subcellular distribution of mammalian PER proteins following coexpression with CKI ϵ or CKI δ seems to depend on the respective PER paralog as well as on the cell line. Although a direct effect of CKI ϵ / δ phosphorylation on subcellular localization can be detected, a consistent direction of this effect is not observed (Takano et al. 2000, 2004; Vielhaber et al. 2000; Akashi et al. 2002).

Phosphorylation regulates inhibitory activity. The activity of many eukaryotic transcription factors (e.g., NFAT, p53, HSF1, CREB, c-Jun, and Fos) is regulated by reversible phosphorylation (Holmberg et al. 2002). As PER proteins are believed to act as transcriptional repressors, it is conceivable that their inhibitory action on CLOCK:BMAL1 could be regulated by phosphorylation, as well. However, direct evidence for this is rare. Only one study demonstrates that phosphorylation of dPER by DBT and CK2 acts in a coordinate manner to potentiate PER repression activity. Importantly, this effect is not mediated by an alteration of the subcellular localization of the protein (Nawathean and Rosbash 2004). Similar results have not been reported in the mammalian system.

Circadian Phenotypes with Altered PER Phosphorylation

How important is the phosphorylation of PER proteins for the generation and dynamics of circadian rhythms? Probably very important, because several severe circadian phenotypes are due to mutations that alter the temporal and spatial phosphorylation patterns of PER proteins. Examples for known circadian phenotypes with altered PER phosphorylation in *Drosophila* and mammals are summarized in Table 1 and described in more detail below.

The first direct evidence of a genetic basis for circadian clocks came from the short- and long-period mutant flies (*per^S* and *per^L*) isolated by Konopka and Benzer (1971). Later, it was shown that in *per^S* mutants, the accumulation profile and nuclear clearance of dPER are advanced (Zerr et al. 1990; Edery et al. 1994), whereas in *per^L* flies, the

nuclear entry of the PER protein is delayed (Curtin et al. 1995). Similar effects were observed in *dbt* mutants (e.g., *dbt^S* and *dbt^L*) (Kloss et al. 1998; Price et al. 1998).

In the mammalian circadian system, the most prominent examples of phosphorylation-based circadian phenotypes are the *tau* mutation in the hamster and the FASPS phenotype in humans. The *tau* mutation was discovered by Ralph and Menaker (1988) on the basis of a prominent shortening of the circadian locomotor activity rhythm—a 22-hour period in heterozygous animals and a 20-hour period in homozygous animals (Ralph and Menaker 1988). The mRNA profiles of *Per1* and *Per2* in the SCN (suprachiasmatic nucleus) of *tau* mutant hamsters show an earlier rise and decline and a reduction in the peak expression levels of both genes (Lowrey et al. 2000; Dey et al. 2005). Genetic analysis revealed that a single-nucleotide exchange in the coding region of the CKIε gene generates the *tau* mutant phenotype. This mutation

Table 1. Circadian Phenotypes with Altered PER Phosphorylation in *Drosophila* and Mammals

Locus/species	Mutant allele	Molecular lesion	Phenotype	Molecular impact	References
period gene, <i>Drosophila</i>	<i>per^S</i>	single-nucleotide exchange leads to Ser-589→Asn	short-period rhythms (19 hr) in eclosion and behavior	abundance and phosphorylation profiles of PER ^S are advanced; premature nuclear clearance, but unaltered nuclear accumulation	Konopka and Benzer (1971); Yu et al. (1987); Zerr et al. (1990); Curtin et al. (1995)
period gene, <i>Drosophila</i>	<i>per^L</i>	single-nucleotide exchange leads to Val-243→Asp	long-period rhythms (29 hr) in eclosion and behavior	nuclear entry and clearance of PER ^L is delayed	Konopka and Benzer (1971); Curtin et al. (1995)
doubletime gene, <i>Drosophila</i>	<i>dbt^S</i>	single-nucleotide exchange leads to Pro-47→Ser	short-period rhythms in eclosion (20 hr) and behavior (18 hr)	accelerated PER accumulation in the cytoplasm and disappearance from the nuclei; reduced kinase activity in vitro	Kloss et al. (1998); Price et al. (1998); Bao et al. (2001); Preuss et al. (2004)
doubletime gene, <i>Drosophila</i>	<i>dbt^L</i>	single-nucleotide exchange leads to Met-80→Ile	long-period rhythms (27 hr) in eclosion and behavior	accumulation pattern of PER is delayed; longer persistence in the declining phase of the circadian cycle; reduced kinase activity in vitro	Kloss et al. (1998); Price et al. (1998); Suri et al. (2000); Preuss et al. (2004)
doubletime gene, <i>Drosophila</i>	<i>dbt^P</i>	P-element insertion in intron 2 disrupts gene function	heterozygous: normal behavioral rhythms homozygous: lethal	hypophosphorylated PER accumulates to high levels in homozygous <i>dbt^P</i> larvae	Kloss et al. (1998); Price et al. (1998)
doubletime gene, <i>Drosophila</i>	<i>dbt^{arr}</i>	single-nucleotide exchange leads to His-126→Tyr	homozygous: arrhythmic behavior	hypo- and hyperphosphorylated PER accumulates to high levels; PER oscillation is stopped	Rothenfluh et al. (2000)
period 2 gene, human	<i>Per2S662G</i> (FASPS)	single-nucleotide exchange leads to Ser-662→Gly	FASPS, ~4-hr advance of circadian parameters, short endogenous period	hypophosphorylation of PER2 protein in vitro; reduced PER2 stability due to decreased nuclear retention	Toh et al. (2001); Vanselow et al. (2006)
casein kinase Iδ gene, human	<i>CKIδT44A</i> (FASPS)	single-nucleotide exchange leads to Thr-44→Ala	FASPS, ~4-hr advance of circadian parameters, short endogenous period	reduced kinase activity in vitro	Xu et al. (2005)
casein kinase Iε gene, hamster	<i>CKIεR178C</i> (<i>tau</i>)	single-nucleotide exchange leads to Arg-178→Cys	<i>tau</i> phenotype: short-period behavioral rhythms heterozygous: 22 hr heterozygous: 20 hr	reduced kinase activity in vitro, premature nuclear clearance of PER1 and PER2 in the SCN; increased phosphorylation of PER proteins in cells	Ralph and Menaker (1988); Lowrey et al. (2000); Dey et al. (2005); Gallego et al. (2006b)

results in an arginine-to-cysteine substitution at amino acid 178 of CKI ϵ . In vitro, recombinantly produced *tau* kinase has a reduced activity, whereas the binding to mPER1 and mPER2 is not impaired (Lowrey et al. 2000). This findings suggest a possible reduction in the phosphorylation of the PER proteins by CKI ϵ (*tau*) also in vivo. Unexpectedly, however, *tau* mutant hamsters exhibit robust circadian rhythms in both PER protein abundance and PER phosphorylation without substantial differences in the degree of hyperphosphorylation, although the overall amount of PER protein is reduced (Lee et al. 2001). Interestingly, an accelerated nuclear clearance of PER proteins in the SCN of *tau* mutant hamsters has been reported (Dey et al. 2005).

A second phosphorylation-based circadian phenotype is the FASPS phenotype in humans. The heredity transmission of FASPS follows an autosomal dominant pattern (Jones et al. 1999; Toh et al. 2001; Xu et al. 2005). Affected individuals are extreme morning larks with an approximately 4–5-hour advance of sleep onset and offset, temperature, and hormonal rhythms. The endogenous period of one affected individual was measured to be 23.3 hours (Jones et al. 1999), which is about 1 hour shorter than the average population (Czeisler et al. 1999). Up to now, genetic analyses have identified two single-nucleotide exchanges in human clock genes correlating with the FASPS phenotype. In one case, the human *Per2* gene is mutated, which leads to a serine-to-glycine exchange at residue 662 of hPER2 (Toh et al. 2001). This region of PER2 is highly conserved within all paralog PER proteins in mammals. Notably, the serine residue mutated in FASPS is the first residue in a cluster of serine and threonine residues forming a canonical CKI ϵ / δ phosphorylation/recognition motif (pS/pT/pYX₍₁₋₂₎S/T; X = any amino acid) (Flotow et al. 1990). In vitro, the FASPS mutation causes hypophosphorylation of PER2 when phosphorylated with CKI ϵ (Toh et al. 2001).

In a second form of FASPS, the human *CKI δ* gene is mutated. Here, a threonine-to-alanine substitution at amino acid 44 in the CKI δ protein has been identified. As in the case of *tau*, recombinantly produced CKI δ -T44A has reduced kinase activity in vitro. Interestingly, transgenic mice containing a human bacterial artificial chromosome (BAC) clone expressing the CKI δ -T44A mutant show a shortened circadian period, whereas the expression of hCKI δ -T44A in flies leads to a period lengthening (Xu et al. 2005), although the respective position is highly conserved between mammalian CKI δ and DBT. This suggests that reducing the activity of CKI δ can result in opposite period phenotypes depending on the cellular context and/or the substrates.

Until recently, the molecular mechanisms of both the *tau* and the FASPS phenotype were postulated as follows: In *tau*, the reduced kinase activity and in FASPS, the lack of a specific phosphorylatable residue in PER2 both result in hypophosphorylation of PER proteins. Because phosphorylation triggers degradation, it has been assumed that PER hypophosphorylation would lead to a stabilization of PER proteins and thus to a more rapid PER accumulation in the cytoplasm, followed by an advanced nuclear localization of PER proteins. An earlier repression of CLOCK-

BMAL1 transcriptional activity then would lead to shorter circadian periods (Lowrey et al. 2000; Dey et al. 2005). However, we propose an alternative mechanism (see below). Although the available data strongly suggest a disturbance of PER phosphorylation as the molecular basis for *tau* and FASPS, direct evidence for altered phosphorylation patterns in vivo was still missing.

Mapping of Phosphorylation Sites in Clock Proteins

The examples described above underline the enormous importance of proper phosphorylation of PER proteins and possibly other clock components for the generation and dynamics of circadian oscillations. The circadian phase-dependent gradual decrease of PER's electrophoretic mobility indicates that several distinguishable PER phosphorylation species exist. In addition, the differential effects of some mutations on PER stability and subcellular localization argue for specific phosphorylation sites influencing these properties. To elucidate how phosphorylation at particular sites regulates the temporal and spatial properties of PER proteins, a detailed map of endogenous PER phosphorylation sites is needed. Knowing these sites would allow for the study of their specific function and may provide hints toward the identification of the kinases responsible for phosphorylation. Previous studies analyzed single phosphorylation sites in mPER1 with respect to their functional roles (Vielhaber et al. 2000; Takano et al. 2004). Until recently, however, no systematic effort to comprehensively map in vivo clock protein phosphorylation sites has been undertaken.

We have established a novel mass spectrometric technique that allows sensitive and comprehensive mapping of protein phosphorylation sites. We use this technique to analyze phosphorylation sites in clock proteins in a systematic manner. Immunoprecipitated proteins are subjected to in-gel digests with a set of four different proteases. Phosphopeptides are enriched on titanosphere columns and then analyzed by ultra-low-flow nanoLC-MS/MS (Schlosser et al. 2005).

The first clock protein that we analyzed was mouse PER2, because it is the best-characterized circadian phosphoprotein to date with a very pronounced temporal phosphorylation profile. For that purpose, we generated a HEK 293 cell line stably expressing carboxy-terminal V5-tagged mPER2 for efficient immunoprecipitation. The mass spectrometric approach mentioned above led to the identification of 21 (of 275 theoretically possible) endogenous phosphorylation sites in mPER2 (exclusively serine and threonine residues). Interestingly, we found the serine residue phosphorylated, which is mutated in FASPS (Vanselow et al. 2006). This is the first direct evidence that the FASPS site is indeed a target for phosphorylation in living cells. Generally, many of the detected phosphorylation sites are located close to the previously identified CKI ϵ / δ -binding domain (amino acids 555–754) (Akashi et al. 2002) or surrounding the nuclear export sequence-2 (NES-2 amino acids 983–990) (Yagita et al. 2002). Further phosphorylation sites are concentrated at the carboxyl and amino termini, and no phosphorylation site was found in the PAS domain region.

To investigate the function of these 21 phosphorylation sites of mPER2, we established a reductionist model system, which is based on the expression of phosphosite-mutated mPER2 proteins in synchronized fibroblasts. Potential alterations in the oscillation properties are analyzed using luciferase-based circadian reporters. Fibroblast cell lines like Rat-1 and NIH-3T3 have been demonstrated to show self-sustained circadian oscillations with properties very similar to those of the SCN-intrinsic cellular oscillator (Balsalobre et al. 1998; Yagita et al. 2001; Brown et al. 2005b). In the first step, we generated an NIH-3T3 Flp-In host cell line harboring the Flp recombinase target (FRT) site (O’Gorman et al. 1991). This ensures the expression of different mPER2 variants from the same chromosomal location in the fibroblast genome. In a second step, expression cell lines of PER2 variants were generated by recombining mutant constructs into the FRT site of the NIH-3T3 genome. Thus, differences in the oscillation dynamics of the resulting expression cell lines or in the biochemical properties of the PER2 variants are directly caused by the PER2 protein mutations, and artifacts due to chromosomal positioning effects or variation of integration events were excluded.

We were particularly interested in investigating the functional role of the PER2 phosphorylation at the FASPS region, because this region has been implicated to be crucial for the development of FASPS. To this end, we generated different variants of mPER2 affecting the FASPS region (FASPS [S659G], mut-7 [S659G, S662A, S665A, S668A, S670A, S671A, TS672A], and S659D) by site-directed mutagenesis. These PER2 variants were then expressed in our NIH-3T3 Flp-In cell line. The circadian oscillations of the resulting cell lines were recorded continuously in a luminometer. Interestingly, the oscillation dynamics of PER2-FASPS expressing cells is very similar to the behavior of FASPS patients: an early phase of entrainment and a short free-running period (Fig. 1) (Vanselow et al. 2006).

Figure 1. Expression of PER2-FASPS causes an advanced phase and a shorter period in oscillating NIH-3T3 fibroblasts after a several day temperature entrainment regime. Real-time circadian oscillations of luciferase reporter activity were recorded in a luminometer. (Reprinted, with permission, from Vanselow et al. 2006.)

Functional Different Phosphorylation Sites in mPER2: A Molecular Basis for FASPS and *tau*

We next wanted to elucidate the molecular processes that are altered in FASPS. In principle, there are several possibilities of how the phosphorylation at the FASPS position and presumably also the following serine and threonine residues of the CKI ϵ / δ phosphorylation cluster might influence the oscillation dynamics. These include the regulation of PER2 stability, PER2 subcellular localization, and/or PER2 inhibition of CLOCK-BMAL1 activity.

We have analyzed all of these possibilities. Alterations in the ability of PER2-FASPS to inhibit *trans*-activation by CLOCK:BMAL1 were not observed. Surprisingly, however, we found that the stability of PER2-FASPS is not—as it might be expected—increased compared to PER2 wild-type protein but is substantially reduced. Although the protein half-life of PER2-wt is about 3 hours, the half-life of PER2-FASPS is reduced to about 1.5 hours. The destabilization is even more pronounced in the PER2-mut-7 variant, in which the whole CKI ϵ / δ phosphorylation cluster is mutated, whereas the stability of the PER2-S659D mutant is not altered (Fig. 2) (Vanselow et al. 2006). Coexpression studies with CKI ϵ revealed that the decreased stability of PER2-FASPS is due to its higher sensitivity toward CKI ϵ -mediated proteasomal degradation. Notably, Takano and colleagues made similar observations when analyzing the corresponding sites in PER1. Coexpression of the PER1 mutant with CKI ϵ resulted in a reduced stability and higher phosphorylation state (Takano et al. 2004; Takano and Nagai 2006). Thus, phosphorylation of PER proteins does not always trigger the degradation pathway but can have differential functions. Although phosphorylation in the FASPS region stabilizes PER proteins, phosphorylation at other residues promotes proteasomal degradation.

Figure 2. The FASPS mutation destabilizes the PER2 protein. NIH-3T3 cells stably expressing indicated PER2 variants were treated for up to 8 hours with the protein translation inhibitor cycloheximide (CHX). The PER2 protein amount was determined by SDS-PAGE/western blotting. (Reprinted, with permission, from Vanselow et al. 2006.)

Next, we wanted to answer the question of why phosphorylation in the FASPS region stabilizes PER2. To this end, we tested whether stabilization is an indirect consequence of an altered subcellular localization of PER2. Although we could not detect a different nuclear import rate of PER2-FASPS, we did observe an accelerated nuclear clearance of the PER2-FASPS protein (Fig. 3)

Figure 3. The FASPS mutation leads to a premature nuclear clearance. PER2 variant expressing NIH-3T3 cells were treated with the protein translation inhibitor cycloheximide (CHX) for the indicated times. Subcellular localization of PER2 proteins was visualized after immunostaining by confocal fluorescent microscopy. (Reprinted, with permission, from Vanselow et al. 2006.)

(Vanselow et al. 2006). This result is similar to observations for PER1 and PER2 in the SCN of *tau* mutant hamsters (Dey et al. 2005). Thus, phosphorylation at the FASPS region increases nuclear retention of PER2, thereby protecting it from cytoplasmic degradation by the proteasome. In the FASPS mutation, however, we propose that the mutant PER2 protein is prematurely exported, which leads to an earlier release of CLOCK-BMAL1 repression and earlier restart of a new cycle (Fig. 4) (Vanselow et al. 2006).

Xu et al. (2007) generated transgenic mice to study the molecular mechanisms underlying FASPS. The protein levels of PER2-FASPS (especially in the nuclear fraction) in the liver of those transgenic mice as well as in fibroblasts from skin biopsies of FASPS patients were found to be reduced as compared to controls. As in our study, this indicates a decreased stability of the PER2-FASPS protein. However, because the authors did not find a stabilizing effect of the proteasomal inhibitor MG132, they excluded the possibility of an altered stability of PER2-FASPS as the primary consequence of the mutation. Instead, they found a reduction of the *Per2* mRNA in FASPS and concluded that phosphorylation at the FASPS position has an impact on *Per2* transcription, a result we did not observe in our model system.

Functionally different phosphorylation sites in PER2 may also—at least qualitatively—explain the molecular mechanism for the *CK1 δ* -based variant of FASPS as well as the *tau* phenotype in the hamster. In contrast to the *Per2*-based version of FASPS, *CK1 δ* -based FASPS and *tau* could be explained by an altered ability of the kinases to phosphorylate PER2 (note, the reduced kinase activity

Figure 4. Functional different phosphorylation sites in PER2 as the molecular basis for FASPS. The PER2 protein contains at least two functionally different phosphorylation sites—one kind primarily mediating proteasomal degradation (green), and the other enhancing nuclear retention (purple). For mechanistic details, see the main text. (Reprinted, with permission, from Vanselow et al. 2006.)

of CKI δ -T44A and CKI ϵ [*tau*] in vitro). Here, the key assumption is that the overall decrease in kinase activity does not lead to an evenly reduction of phosphorylation at all PER2 phosphorylation sites but to a specific decrease or inability to phosphorylate specific residues in the protein. According to our model, both CKI δ -T44A and CKI ϵ (*tau*) are impaired in the phosphorylation of sites, which stabilize PER2 (maybe even at the FASPS region), whereas the phosphorylation at destabilizing degradation-promoting sites is unaffected or less affected. This may lead to a premature nuclear clearance of PER proteins and therefore to the observed early phase and short period of the FASPS individuals or *tau* hamsters, respectively.

To conceptualize our results, and to better understand their implications for circadian rhythm generation in a more quantitative manner, we constructed a mathematical model of PER2 phosphorylation in the circadian system (Vanselow et al. 2006). The model shows robust self-sustained oscillations with a period of 24 hours and delay of 6 hours between the *Per2* mRNA and PER2 protein rhythms. Furthermore, the simulation of an intervention with phosphorylation events that promote nuclear retention produces a short period and early phase as observed for FASPS patients. The model was also successfully applied to deduce testable predictions for the effects of specific system perturbations. For example, one biochemical prediction from the mathematical model is that the coexpression of PER2-wt with CKI ϵ (*tau*) or CKI δ -T44A should destabilize the PER2-wt protein to a higher degree than the coexpression of PER2-wt with CKI ϵ (wt). Concomitantly, the destabilizing effects of CKI ϵ , CKI ϵ (*tau*), and CKI δ -T44A on PER2-FASPS should be similar to each other. We tested these predictions for the *tau* kinase in cultured cells, and as predicted, the destabilizing effect of CKI ϵ (*tau*) on PER2-wt was much more pronounced than that of the wild-type kinase. Furthermore, the destabilizing effect of CKI ϵ (*tau*) on PER2-wt and PER2-FASPS was comparable.

In line with these results, Gallego et al. (2006b) found a higher destabilizing effect of the *tau* kinase on PER1 and PER2 in cell culture. The starting point of their experiments was mathematical modeling, which claimed an increased kinase activity (gain-of-function) for CKI ϵ (*tau*) in vivo as the only possible explanation for the short-period phenotype. With respect to the *tau* mutation, our model and that of Gallego et al. (2006b) offer alternative but not mutually exclusive explanations. We favor a mechanism where a reduced kinase activity of mutant CKI ϵ leads to an altered, less nuclear localization of PER proteins (as observed by Dey et al. 2005). Because phosphorylation-induced proteasomal degradation of PER proteins is primarily a cytosolic event (Vanselow et al. 2006), the overall phosphorylation of the PER proteins in *tau* hamsters may even increase (as observed by Gallego et al. 2006b). We propose that this increased phosphorylation is primarily due to a higher amount of PER substrate in the cytoplasm, rather than to an increased kinase activity. Nevertheless, the available data on the *tau* mutation fit both models. With respect to the *Per2*-based FASPS mutation, however, the cellular localization data (Vanselow et al. 2006) strongly favor the localization-dependent model that we postulate.

CONCLUSIONS

Experimental results from the last two decades underline the crucial importance of posttranslational mechanisms for both the generation and dynamics of circadian rhythms. Specifically, the reversible phosphorylation of the PER proteins in *Drosophila* and mammals seems to have a key role for the correct timing of nuclear repression. Animals with altered PER phosphorylation (by either a mutant PER protein or a mutant kinase) have striking circadian phenotypes at the behavioral as well as the molecular level. Biochemical analyses indicate that phosphorylation regulates not only the stability of PER proteins, but also their subcellular localization and inhibitory activity. Until recently, it was unknown which of the many potential phosphorylation sites of PER proteins are functionally relevant in vivo. Our aim is to perform a comprehensive phosphorylation function analysis of clock proteins. For that purpose, we developed an optimized mass spectrometric approach and were able to identify endogenous phosphorylation sites of mPER2. In a cell culture model system, we then studied the oscillation dynamics of cells expressing phosphosite-mutant PER proteins. This approach allowed us to analyze the molecular basis for the human FASPS. Remarkably, the phosphorylation site in PER2, which is mutated in FASPS, stabilizes the protein by means of nuclear retention and thus protects it from cytosolic degradation. On the basis of our data, we proposed a model of functionally different phosphorylation sites in PER2, which is also able to explain additional phosphorylation-based circadian phenotypes. We are currently investigating the role of other identified phosphorylation sites, especially with respect to potential destabilizing effects.

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