BACULOVIRAL AND MARSUPIAL CPD PHOTOLYASES: DNA REPAIR PROTEINS WITH A CIRCADIAN CLOCK FUNCTION

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Magdalena A. Biernat

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Abstract

Baculoviruses infect insects and are highly virulent, host specific and environmentally safe, and therefore, are used as biocontrol agents of pest insects. Their effective use in the field is hampered, however, by the ultraviolet (UV) light, which induces cyclobutane pyrimidine dimers (CPDs) in (viral) DNA. CPD photolyases are enzymes that repair CPDs with the help of visible light in a process called photoreactivation. The baculovirus *Chrysodeixis chalcites* nucleoplyhedrovirus (ChchNPV) possesses two photolyase genes, *Cc-phr1* and *Cc-phr2.* Only *Cc-phr2* encodes an enzymatically active photolyase. CPD photolyases are members of the cryptochrome/photolyase family (CPF), which consists of two types of proteins that are structurally conserved, but have different functions. Whereas photolyases repair DNA, cryptochromes work as photoreceptors or regulators of the circadian clock. In mammals, cryptochromes act as inhibitors of CLOCK/BMAL1-driven transcription, and hence, regulate proper functioning of the 24h oscillator. This thesis focuses on the origin and function of baculovirus CPD photolyases. Phylogenetic analysis indicated that a single horizontal gene transfer from an ancestral lepidopteran host led to the current presence of *phr* genes in a particular clade of the family *Baculoviridae*. A next study examined whether ChchNPV occlusion bodies (OBs) profited from the photolyases in becoming less UV sensitive. However, this was not the case, as the OBs's DNA could not be photoreactivated after UV irradiation. This led to the hypothesis that Cc-PHRs may have another function in baculovirus pathogenesis, or alternatively, may induce behavioral changes in the host. Considering the homology to cryptochromes, a possible role of CPD photolyases in the molecular oscillator ('clock') was studied. This revealed that Cc-PHR2 and the *Potorous tridactylus* photolyase *(Pt*CPD-PL), in contrast to Cc-PHR1 and *Arabidopsis thaliana* (6-4) photolyase, were able to substitute for mammalian cryptochromes. Both Cc-PHR2 and *Pt*CPD-PL inhibited CLOCK/BMAL1-driven transcription and dampened the oscillation of cultured fibroblasts, probably due to the observed interaction with the CLOCK protein. Moreover, both CPD photolyases revived oscillations in arrhythmic cryptochrome knockout (*Cry1*$^{-/-}$*/Cry2*$^{-/-}$) mouse dermal fibroblasts and livers, showing that Cc-PHR2 and *Pt*CPD-PL can work as true cryptochromes. Chromatin- and co-immunoprecipitation experiments showed that Cc-PHR2 and *Pt*CPD-PL do not prevent CLOCK to bind chromatin nor do they disrupt CLOCK/BMAL1 heterodimer formation. Therefore, these proteins probably use a similar mechanism as mammalian cryptochromes to drive the clock and their overall structure rather than their amino acid sequence and/or domain availability determines their functionality. The gathered data advanced our understanding of the functional evolution of the CPF and showed that the studied class II CPD photolyases are dually functional proteins that repair DNA, but can also regulate the circadian clock in a similar manner as cryptochromes.

Key words: photolyases, cryptochromes, circadian clock, DNA repair, functional evolution, baculoviruses

Contents

CHAPTER 1

General introduction

Baculoviruses

Baculoviruses infect invertebrates, predominantly the insects of the orders Lepidoptera, Hymenoptera and Diptera (Martignoni et al., 1981; Blissard, 1996). The *Baculoviridae* family consists of large rod-shaped, enveloped viruses with a circular double-stranded DNA genome with a size of 80-180 kilobasepairs (kbp) (reviewed by van Oers and Vlak, 2007). The genomes of baculoviruses are packaged in bacilliform-shaped nucleocapsids and wrapped into an envelope (Miller, 1997b; Rohrmann, 2008). The shape of the virions determined the name "baculovirus" for this type of virus (from Latin, baculum = rod).

Baculoviruses are transmitted in the form of occlusion bodies (OBs) that immobilize the virus within a paracrystalline protein matrix, assuring survival within the insect host during decomposition of the host cells at the end of infection and protecting it against environmental conditions while awaiting a new host to infect. Based on occlusion body (OB) morphology, baculoviruses can be divided into (i) nucleopolyhedroviruses (NPV), when multiple virions are occluded in a matrix of the protein polyhedrin, and (ii) granuloviruses (GV) when occluded singly into a granulin matrix (Ackermann and Smirnoff, 1983). The OB immobilizes the virus within the paracrystalline protein matrix, protecting it against environmental conditions and assuring survival within the insect host during decomposition of the host cells and at the end of infection. Whereas GV virions contain only one nucleocapsid per envelope, NPVs contain either single (SNPV) or multiple (MNPV) nucleocapsids per envelope (Figure 1) (Slack and Arif, 2007). The taxonomy of the *Baculoviridae* recognises four genera. The viruses that infect Lepidoptera are divided into Alphabaculoviruses and Betabaculoviruses, which encompass NPVs and GVs, respectively. The NPVs that infect insects in the order Hymenoptera and Diptera are named Gammabaculoviruses and Deltabaculoviruses, respectively (Jehle et al., 2006).

ODVs versus BVs and the infection cycle

Baculoviruses in general have two virion phenotypes: occlusion-derived virions (ODV) and budded virions (BV). Although these two types of virions are similar in their nucleocapsid structure and DNA content, they are different in origin and composition of their envelopes, and they perform different roles in the virus infection cycle (Figure 1 and Figure 2). ODVs are responsible for the primary infection of midgut epithelial cells, whereas BVs are responsible for secondary systemic infections within the insect body (reviewed by Slack and Arif, 2007; Rohrmann, 2008; Szewczyk, 2008).

The infection is initiated when the insect host feeds on a plant that contains the baculovirus in the form of OBs (Figure 2). ODVs are released from OBs upon encountering the alkaline environment of the insect's midgut. The primary infection starts when ODVs, after passing the peritrophic matrix (Hegedus et al., 2009) that lines the gut, fuse to the membrane of the microvilli of columnar epithelial cells (Granados and Lawler, 1981) and the nucleocapsids enter the cell. Subsequently, the nucleocapsids are transported into the nucleus via actin filaments (Lanier and Volkman, 1998). For the midgut infection process several structural ODV proteins, including the peroral infectivity factors PIF1, PIF2, PIF3, PIF 4, PIF 5 and P74 are essential. These four proteins form a complex on the ODV surface (Haas-Stapleton et al., 2004; Ohkawa et al., 2005; Peng et al., 2010; Pijlman et al. 2003, Fang et al., 2009, Sparks et al., 2011). The mechanism of the transport within the cell is not entirely known. It is likely, though, that nucleocapsids use myosin VI and actin-binding for transport through the microvilli (reviewed by Volkman, 2007).

Nucleocapsids finally enter the nucleus through nuclear pores (Granados, 1978). Viral DNA transcription, replication, and assembly of nucleocapsids occurs in the nucleus and BVs are produced by budding from the cells at the basal lamina lining the midgut and connective tissues to be transported through hemolymph and trachea to initiate secondary infections in other body tissues (Rohrmann, 2011a).

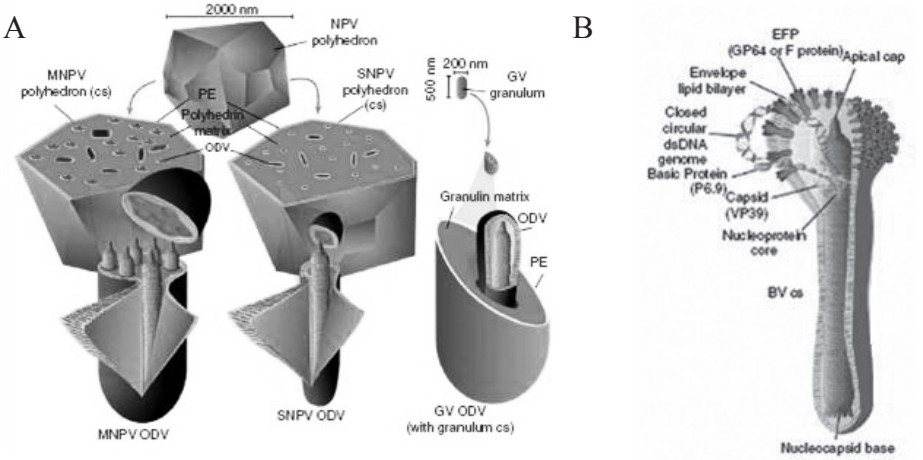

Figure 1. (A) Structure of occlusion body and occlusion-derived virus (ODV) of both Nucleopolyhedroviruses (NPVs) and Granulovirus (GV); (B) budded virus (BV) with indicated major fusion proteins (EFPs) and structural components (adapted from Slack and Arif, 2007).

In the cell, the infection process can be divided into four time phases, but the exact timing varies with the virus, the host and the temperature. The immediate-early phase for *Autographa californica* (Ac) MNPV occurs approximately from 0-2 h post infection (p.i.) and is under control of host RNA polymerase II. The late phase is estimated between 6 and 24 h p.i. and involves replication processes and expression of predominantly virion proteins prior to assembly and the very late phase up to 72 h p.i. when ODVs are occluded and OBs are formed (Rohrmann, 2011b).

In the cell nucleus, ODVs are then wrapped in de novo synthesized envelopes (Hong et al., 1997). Those ODVs are subsequently embedded in either a polyhedrin or granulin matrix to form OBs. As a consequence of the infection, the whole insect body finally undergoes liquefaction (wilting disease), mediated by viral-encoded chitinase and cathepsin (Hawtin et al., 1997) and the OBs are released into the environment awaiting a new host (Figure 2).

Baculovirus Evolution

The most accepted evolutionary scenario is that baculoviruses have evolved from non-occluded viruses that infected only the midguts to occluded viruses that infect midguts (gamma- and deltabaculoviruses) and finally to occluded viruses with the ability to spread the infection to other tissues (alpha- and betabaculoviruses) (Herniou and Jehle, 2007). It is also accepted that during evolution baculoviruses obtained the ability to spread infection to different cell types and became more and more independent from the host cell machinery. Baculoviruses as a group are genetically highly variable as a result of extensive recombination events within the baculovirus genome, horizontal gene transfers (among baculoviruses or baculoviruses and their hosts), transposition

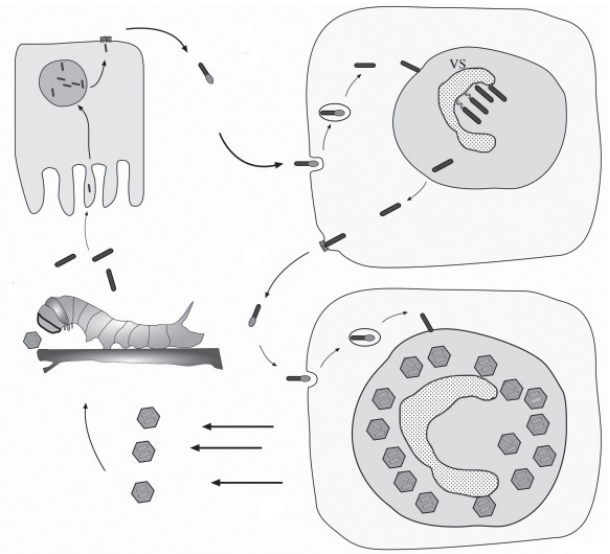

Figure 2. The baculovirus infection cycle. OBs are taken up orally by the larvae and dissolve in the alkaline environment of the midgut. When ODVs are released, they infect epithelial midgut cells. Virus replicates and budded viruses (BVs) are produced and systemic infection begins, which finally leads to accumulation of occlusion bodies in other tissues, e.g. fat body. At the late stage of infection, the larval body disintegrates and new OBs are released (adapted from Rohrmann, 2008).

events, gene insertions and deletions (Jehle et al., 1998, Herniou et al., 2007). Baculovirus evolution was probably shaped by the environment (part of their infection cycle includes persistence outside the host) and by coevolution with the insect host (Hughes and Friedman, 2003).

Baculovirus evolution, in relation to their hosts, has been analysed several times and different hypotheses have been made (Rohrmann, 1992). The most plausible evolutionary scenario postulates that the phylogenetic separation of the baculoviruses into clusters follows the classification of the hosts (Herniou et al., 2004). This scenario is based on the idea that different hosts applied selective pressure on baculoviruses, which led to the specialization of baculoviruses to the specific insect hosts. According to current phylogenetic analyses, baculoviruses are classified into at least four evolutionary lineages: the most ancestral dipteran NPVs, the hymenopteran NPVs, and the lepidopteran NPVs and GVs (Herniou and Jehle, 2007). The degree of evolutionary relatedness among these viruses can be further discerned, with lepidopteran NPVs divided into two groups (group I and group II), and two distinct sub-groups among group I viruses (clade 1a and clade 1b) (Jehle et al., 2006; Zanotto et al., 1993).

Usage of baculoviruses in biocontrol and biotechnology

Baculoviruses are being used both in biocontrol of pest insects and in biotechnology. First, their potential as biopesticides was exploited (since the 1920s) to protect field crops (GemStar™), forests (Gypchek™, Virin-ENSH™) and greenhouse plant protection (SPOD-X™) and is described in detail in the next paragraph. Later, they were also recognized as efficient vectors for recombinant protein production in insect cell cultures (since the early 1980s) to express a range of recombinant proteins in insect cells, to produce virus-like particles and virus vectors (Smith et al., 1983; van Oers et al., 2006; Hitchman et al., 2009). The baculovirus-insect cell expression system is being used to produce commercial veterinary and human vaccines (e.g. against classical swine fever (CSF, Intervet International), influenza virus infection (FluBlok, Protein Sciences) or cervical cancer (Cervarix ™ GSK) (Depner et al., 2001; Cox and Hollister, 2009; Trimble

and Frazer, 2009). Insect cells coinfected with recombinant baculoviruses are being used, additionally, to produce infectious adeno-associated virus (AAV) vectors for gene therapy purposes (Urabe et al., 2002). Recently, the role of BEVS became even broader as it was shown that baculovirus vectors could be used to transduce many mammalian cell lines, thereby providing a tool for gene delivery and thus pharmaceutical screening, cancer therapy, tissue engineering or as a gene therapy vector (reviewed by Hu, 2006; Hitchman et al., 2011; Lesch et al., 2011 Rivera-Gonzalez et al., 2011). The baculovirus expression vector system (BEVS) is nowadays one of the most extensively used tools in biotechnology.

Baculoviruses for pest control

Phytophagous pest insects are a major problem in agriculture and a serious threat for food, flower and wood production. There is a need, therefore, for usage of pesticides. Nowadays, the development of resistance of insects against chemical insecticides and the prohibition to use some insecticides due to their risk for human health and the environment has lead to investigations towards developing alternative (bio)pesticides. Among these are baculoviruses, which can be used as natural biocontrol agents for pest insects as they have a narrow host specificity and are environmentally safe (Black et al., 1997).

A variety of baculoviruses have been applied in biocontrol since the 1930s and several of these viruses have been commercialized for insect control products for: (i) field crops e.g. GemStar™, which is based on *Helicoverpa zea* SNPV and controls pests belonging to the genera *Helicoverpa* and *Heliothis* and Mamestrin™ (based on *Mamestra brassicae* MNPV), which is used on cabbage, tomatoes and cotton to control the cabbage moth (*Mamestra brassicae*), American bollworm (*Helicoverpa armigera*), diamondback moth (*Plutella xylostella*), potato tuber moth (*Phthorimaea operculella*) and grape berry moth (*Paralobesia viteana*); (ii) forests e.g. *Lymantria dispar* MNPV (Gypchek™, Virin-ENSH™), *Orgyia pseudotsugata* MNPV (TM-BioControl-1™ and Virtuss™); (iii) greenhouse plant protection e.g. SPOD-X™, based on *Spodoptera exigua* MNPV is used to control *S. exigua* larvae (Smits and Vlak, 1994; Kolodny-Hirsch et al., 1997). However, there is need to enhance the effectiveness of these biocontrol products by improving virulence and accelerating killing speed (Inceoglu et al., 2001; Bonning and Hammock, 1996). To accelerate the "time-to-kill", baculoviruses were engineered to express insecticidal neurotoxins, hormones or growth regulator genes (Sun et al., 2002; Miller et al., 1997a; Popham et al., 1997; Hughes et al., 1997). The speed of action can also be accelerated by deleting the viral ecdysteroid UDP-glucosyltransferase (*egt*) gene, which inhibits larval molting and prolongs host survival time (Cory et al., 2004).

The use of baculoviruses as biocontrol agents in the field is hampered as well by their susceptibility to UV light (Jaques, 1985). Therefore, UV protectant agents are added to baculovirus formulations (Black et al., 1997). The UV protectants are costly and not efficient enough as can decrease the ability of baculovirus to adhere to the surface of the plant (Black et al., 1997) and may, more importantly, compromise the use of baculoviruses in biological or organic farming systems. The UV sensitivity has limited the application of baculovirus as insecticides in the field and challenges the researcher to look for alternative strategies.

Sunlight and UV damage

Life on earth depends on the availability of sunlight as it is the main source of energy for photosynthesis in plants, and a signal for development and locomotion that is recorded by numerous biological photoreceptors. Light is synchronizing circadian and seasonal rhythms as it is the strongest environmental cue that transmits a signal to adjust the behaviour, metabolism and physiology to the 24-hour rotation cycle of the globe and the seasonal changes on earth. Simultaneously, the ultraviolet (UV) part of the spectrum, when absorbed by DNA, can induce two types of helix-distorting DNA lesions, where two adjacent pyrimidine bases, usually thymines, are linked by two covalent bonds: *cis-syn*-cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6-4)-pyrimidone photoproducts (6-4)PPs (reviewed by Brettel and Byrdin, 2010). The number of CPDs induced by UV light is three-fold higher than the number of (6-4)PPs (Essen and Klar, 2006). Possible consequences of DNA distortion are errors in transcription and replication, which trigger senescence, premature aging or apoptosis. Moreover, this may induce irreversible mutations that can result in carcinogenesis (Mitchell et al., 2003). Organisms exposed to sunlight require, therefore, efficient DNA repair systems. In nature, systems have evolved that are often selective for a specific DNA lesion.

DNA photolyases

Photolyases are proteins that can repair the UV-induced damage in double-stranded DNA via a light-dependent, ATP-independent mechanism, called enzymatic photo-reactivation. Two types of photolyase exist, which are distinguished based on the substrate-specificity. CPD photolyases can repair CPDs, whereas (6-4)-photolyases can repair (6-4)PPs (reviewed by Müller and Carell, 2009; Eker et al., 2009). Photolyases occur in all organisms except placental mammals, which have lost their photolyase genes during evolution and have to rely on a single mechanism to repair CPDs and (6-4)PPs, more specifically Nucleotide Excision Repair (NER) (Yasui et al., 1994, Hoeijmakers, 2001). In spite of the fact that photolyases are not present in placental mammals, enzymatic functionality of exogenous photolyases against DNA lesions was shown in mice. It was shown that both *Arabidopsis thaliana* (6-4)-photolyase or the *Potorous tridactylus* (rat kangaroo) CPD photolyase are functional when expressed in mice and efficiently remove UV-induced lesions in a light-dependent manner (Schul et al., 2002; Jans et al., 2005, 2006).

Based on amino acid divergence, two classes of CPD photolyases are distinguished (Kanai et al., 1997). Class I photolyases occur in most microbial or ganisms, whereas class II photolyases occur in eukaryotes, several bacteria, and some viruses including poxviruses and baculoviruses (O'Connor et al., 1996; Bennett et al., 2003; van Oers et al., 2004).

CPD photolyases are, according to the crystal structure of several class I CPD photolyases, globular monomeric proteins with a molecular mass ranging from 45 to 66 kilodalton (kDa), that carry two cofactors: the redox active flavin adenonucleotide (FAD) and an antenna pigment, which is either 5,10-methylentetrahydrofolate (MTHF) in e.g. *Escherichia coli* or 8-hydroxy-7,8-didemethyl-5-deazariboflavin (8-HDF) in e.g. *Aspergillus nidulans* photolyase (Park et al., 1995; Tamada et al., 1997).

Repair mechanism of CPD photolayses

The CPD is flipped out of the DNA-helix in order to form a stable complex with the

photolyase enzyme and to enter its CPD binding site, after which the photolyase recognizes the lesion (Figure 3). Substrate binding is rapid and highly effective. The photolyase-CPD lesion complex becomes stabilized by hydrogen bonds between the protein and atoms in the dimer, by a tryptophan wedge around the dimer and by salt bridges between the photolyase and phosphates in the DNA backbone (Mees et al., 2004). The antenna cofactors absorb blue/near UV photons and transfer that excitation energy to $FADH^-$. The excited state ($FADH^-$*) transfers an electron to the T=T dimer (Figure 3). That reaction results in a dimer anion radical and neutral flavin radical FADH°. The C5-C6' and C6-C6' bonds split and one thymine and one thymine anion radical are created. The thymine radical transfers its electron excess to FADH° restoring FADH° to $FADH^-$ and with two intact thymines the photoreactivation cycle is finished (Figure 3). The CPD splitting mechanism and details of the electron transfer have not been entirely unraveled, in contrast to the electron transfer during repair (reviewed by Brettel and Byrdin, 2010; Sancar, 2003).

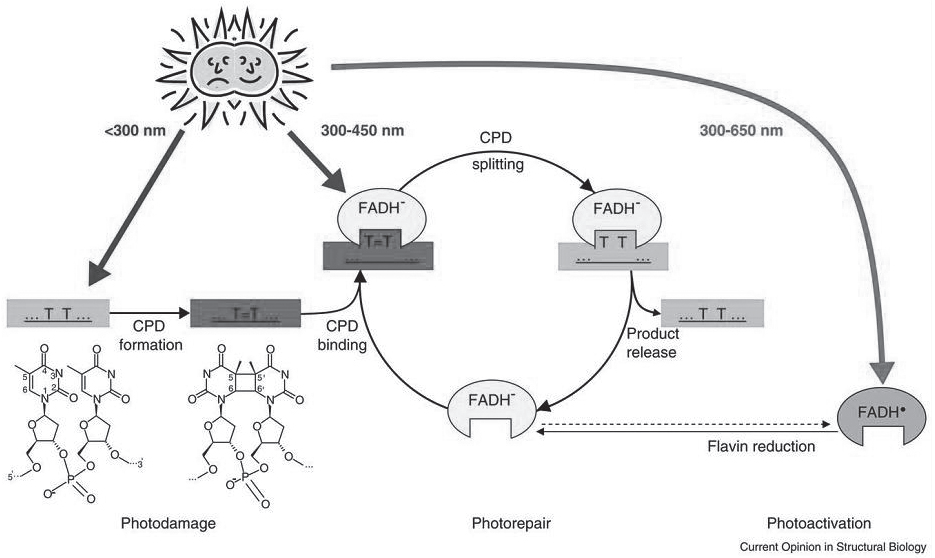

Figure 3. Light reactions relevant for CPD photolyase and enzymatic photoreapair of CPD lesions. Green and red boxes indicate intact and damaged DNA, respectively. T=T is a CPD formed from two thymines. Yellow and blue ellipses indicate photolyase with flavin in fully reduced ($FADH^-$) and semi-reduced state (FADH°), respectively (adapted from Brettel and Byrdin, 2010).

Baculovirus photolyases, UV and behavioral changes

Up till now, 55 baculoviral genomes have been sequenced, but only a few baculoviruses were found to have a photolyase gene (Harrison, 2009; van Oers et al., 2005; Willis et al., 2005). Upon infection by a baculovirus, lepidopteran larvae become hypermobile and climb up the foliage (Kamita et al., 2005). This change in behavior has first been described as "tree-top disease" or "wipfelkrankheit" (Goulson, 1997). That behavior is thought to enhance the spread of progeny virus over the plant and to increase the chance of virus transmission to the next host insect. At the same time, the exposure of the virus to UV light will increase, and can lead to inactivation of progeny virus (Sun et al., 2004). In that perspective possession of a photolyase, an enzyme that repairs UV-caused

DNA damage, could be advantageous for survival of a baculovirus. The first baculovirus CPD photolyases (*phr)* genes were found in *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) and in *Trichoplusia ni* (Tn) SNPV (van Oers et al., 2004, 2005; Willis et al., 2005). This observation was followed by demonstrating that all tested alphabaculovirus group II NPVs, infecting lepidopteran insects from the Plusiinea subfamily of the Noctuidae contain one or more *phr* genes encoding class II CPD photolyases (Xu et al., 2008). A *phr* gene was also found in the betabaculovirus *Spodoptera litura* granulovirus (SpliGV) (Wang et al., 2008), which is only distantly related to the plusiine baculoviruses.

ChchNPV possesses two photolyases genes named *Cc-phr1* and *Cc-phr2.* The Cc-PHR1 and Cc-PHR2 proteins share 45% identity at the amino acid level (van Oers et al., 2005). Phylogenetic analysis has shown that baculovirus *phr* genes form a single clade indicative of a common ancestor, but the origin of the baculoviral *phr* and relationship between baculoviral and insect *phr* genes remains enigmatic. The Cc-PHR2 protein, in contrast to Cc-PHR1 protein, was found to be able to complement an *E. coli* photolyase deficiency and could repair T-T dimers *in vitro*. It was proven, therefore, that the Cc-PHR2 protein has photolyase activity *in vitro* (van Oers et al., 2008). Preliminary experiments have indicated that when introducing the *phr2* open reading frame under control of the AcMNPV *ie-1* promoter into the *Helicoverpa armigera* NPV genome, a decrease in UV sensitivity of this virus after UV treatment and photoreactivation was observed (Xu, 2010c).

According to transient expression assays in insect cells Cc-PHR1 and Cc-PHR2 are targeted to the cell nucleus and are able to bind DNA (Xu et al., 2010). Cc-PHR2 can be found, additionally, in the virogenic stroma of the nucleus upon AcMNPV infection in T.ni cells. Both photolyases were not detected, however, in the ChchNPV ODV particles by LC/MS-MS (Xu et al., 2011) suggesting they have a function not in the OB but in the infected cell nucleus, e.g. at replication.

Cryptochrome /photolyase family and evolution of cryptochrome /photolyase family members

Photolyases belong to the cryptochrome/photolyase family (CPF) of flavoproteins. Cryptochromes are structurally and sequence-wise related proteins that do not have a DNA repair activity, but function as blue-light receptors, circadian photoreceptors, or transcriptional repressors in the circadian clock, and were recently shown to participate in magnetoreception (Cashmore et al., 1999; Kume et al., 1999; van der Horst et al., 1999; Cashmore et al., 2003; Gegear et al., 2010; Ritz et al., 2000). A new class of CPF proteins with CPD photolyase activity for single-stranded DNA (in contrast to the class I and II CPD photolyases mentioned above which recognize lesions in double-stranded DNA) was recently identified in the bacteria *Synechocystis (*Hitomi et al., 2000) and *Vibrio cholerae* (Worthington et al., 2003), in *Arabidopsis thaliana* (Kleine et al., 2003), in *Xenopus laevis, Drosophila melanogaster* and *Homo sapiens* (Brudler et al., 2003; Daiyasu et al., 2004) and termed Cry-DASH for cryptochrome - *Drosophila*, *Arabidopsis*, *Synechocystis*, Human. Cry-DASH proteins show, however, a higher sequence homology to *Drosophila* and human cryptochromes than to bacterial photolyases (Hitomi et al., 2000; Brudler et al., in 2003; Daiyasu et al., 2004, Pokorny et al., 2008).

Cryptochromes are strikingly similar in protein architecture to photolyases by sharing a conserved core domain of about 500 amino acids, the Cryptochrome/ Photolyase Homology Region, which binds FAD and either MTHF or 8-HDF cofactors

(Figure 4). Cryptochromes do not possess, however, the N-terminal extension, which contains the nuclear and mitochondrial localization signals characteristic for photolyases (Sancar, 1994; Cashmore, 1999). On the contrary, cryptochromes (with the exception of Cry-DASH) are equipped with an additional C-terminal extension of varying length and amino acid composition, which harbours a nuclear localization signal (Daiyasu et al., 2004; Chaves et al., 2006; reviewed by Eker et al., 2009). The proposed theory is that the functional diversity between members of the CPF is due to presence or absence of this C-terminal extension and the variety within this domain (Chaves et al., 2006).

Phylogenetically, the CPF is divided into two groups: the first contains class I CPD photolyases, (6-4)PP photolyases, Cry-DASH and both animal and plant cryptochromes, whereas the second group contains only class II CPD photolyases (Kanai et al., 1997). The common hypothesis is that the ancestor of this family is a photolyase that offered protection against UV irradiation and that the photoreceptor function characteristic for today's cryptochromes evolved later, allowing early metazoans to recognize circadian changes and avoid harmful UV light by descending in the oceans during daytime. It was postulated, thus, that the UV component of sunlight contributed to the selective pressure for the evolution of the specialized photoreceptor cryptochrome from photolyases involved in DNA repair (reviewed by Gehring and Rosbash, 2003). It was suggested further that the blue-light specificity of photoreception evolved in an aquatic environment as only blue light can penetrate to substantial depths in water (Cashmore et al., 1999; reviewed by Gehring and Rosbash, 2003). Phylogenetic analysis has further shown that by two independent events animal cryptochromes evolved from (6-4)PP photolyases, whereas plant cryptochromes evolved from CPD photolyases (Cashmore et al., 1999). The recent characterization of Cry-DASH showed that cryptochromes must have first appeared before the divergence of prokaryotes and eukaryotes (Brudler et al., 2003; Kleine et al., 2003).

Fusion of the C-terminal domain of mCRY1 (aa 371-606) to *Arabidopsis thaliana* (6-4)PP photolyase allowed the chimeric protein to inhibit CLOCK/BMAL1 driven transcription (detailed in the next paragraph) (Chaves et al., 2006). That allows suggesting that the remainder of the photolyase/cryptochrome core domain is very important for the clock function of cryptochromes, likely through a complex network of interactions and core domain structure for the proper transcription inhibition (Chaves et al., 2006). Recently, (6-4)PP photolyases were tested for their partial mammalian cryptochrome repressor function in circadian clock shedding light on evolution of CPF and functional diversity between cryptochromes and photolyases (Coesel et al., 2009; Heijde et al., 2010). All those suggest that it is possible to have photoreactivating and clock repressor function in the same protein.

The circadian clock

The circadian clock is found across all three kingdoms of life (Planta, Animalia and Prokaryotes) to help organisms to adapt to the day night cycles (light-dark L/D cycles) as imposed by the rotation of the earth. The term *circadian* means that the length of one period is *circa* one day (from Latin *circa* =about, *dies* = day)). The circadian rhythms are generated endogenously with a period of about 24 h and the circadian clock regulates the physiology, metabolism and behavior of organisms (Pittendrigh, 1960). To keep pace with L/D cycles, the circadian clock has to be adjusted every day, and the strongest "Zeitgeber" (from the German, "time giver") is visible light. Circadian rhythms can be

defined by a few common characteristics: (i) they are persistent in the absence of any environmental cues and so are self-sustained, (ii) they are temperature-independent, and (iii) they can be entrained as they can track environmental changes (Pittendrigh, 1960). In short, a circadian clock is composed of three main parts that can be found in all circadian systems studied so far: a central clock, which generates internal body time, an input that keeps the clock synchronized with environmental cues, and an output that couples the clock to physiological and behavioral mechanisms. In mammals, th e master clock is located in the suprachiasmatic nuclei (SCN) in the brain at the base of the hypothalamus. SCN receive light-resetting signals transmitted from the eyse's retina

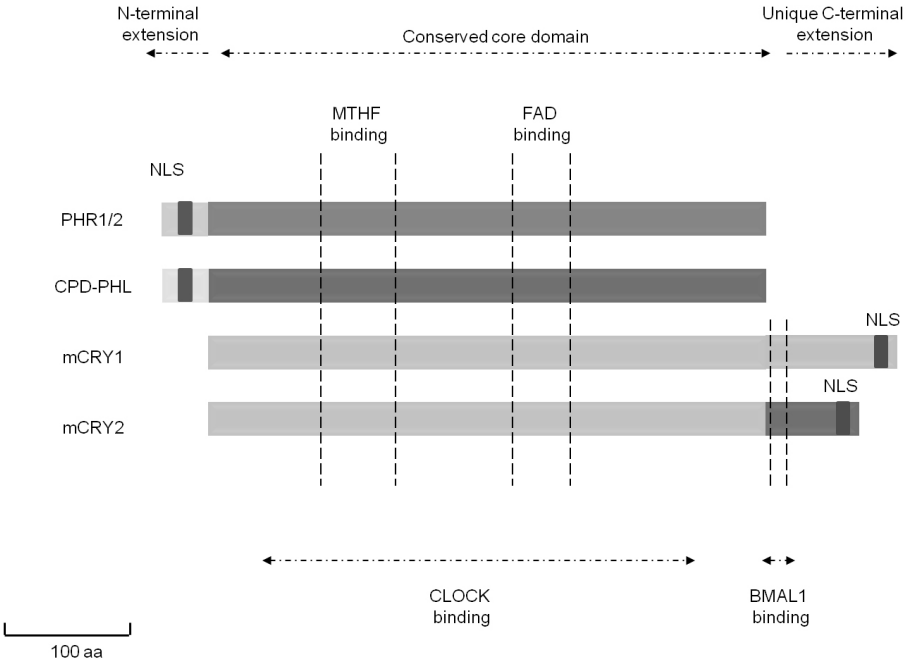

Figure 4. Domain structure of cryptochromes (mCRY1 and mCRY2) and photolyases (PHR1, PHR2 and CPD-PHL). The core domain binding two light-absorbing chromophores, methenyltetrahydrofolate (MTHF)/HDF and flavin adenin dinucleotide (FAD), is highly conserved among all classes of cryptochromes and photolyases. The C-terminal domains containing nuclear localization signals (NLS) are of variable length and poorly conserved, even among cryptochromes of the same species (e.g., mCRY1 and mCRY2). These C-terminal extensions are not found in photolyases, which possess an N-terminal extension containing mitochondrial and nuclear localization signals (NLS).

(Rusak, et al., 1989) and in turn send humoral and neuronal signals that will synchronise the peripheral oscillators, located in various cells and tissues (Balsalobre et al., 1998; Pando et al., 2002).

Extensive studies on the circadian clock of many model organisms have shown that circadian rhythms are generated by a molecular oscillator that is based on transcription-translation feedback loops (TTFLs) in which, with a periodicity of approximately 24 h, clock proteins regulate expression of the genes encoding these clock proteins (Dunlop et al., 2007; Loros et al., 2007) (Figure 5). Next to transcription/ translation feedback loops, clock proteins are subjected to post-translation modifications that assure precision and robustness of the oscillator: phosphorylation, acetylation, sumoylation and ubiquitylation (Kivimae et al., 2008; Fang et al., 2007; Lee at a., 2001;

Yagita et al., 2002; Asher et al., 2008; Siepka et al., 2007).

The work described in this thesis correlates to insect and mammalian clock systems and therefore the molecular mechanism behind these systems is further discussed in the next section.

The molecular oscillator generates circadian rythms

The first experiments to unravel a genetic basis for the circadian clock were performed with mutant *D. melanogaster* fruit flies, which were found to have abnormal behavioral phenotypes with shorter or longer clock periods or which were arrhythmic. Analysis of the mutations resulted in mapping the *Period* (*Per*) locus (Konopka and Benzer, 1971).

The first mammalian (murine) locus found to regulate the circadian oscillator was *Clock* (Circadian Locomoter Output Cycle Kaput) (Vitaterna et al., 1994). In the mammalian clock, in the positive limb of the feedback loop, the CLOCK protein together with BMAL1 (Brain and Muscle Arnt-like protein 1) form a heterodimer that binds to E-box elements in the promoters of *Crypotochrome* (*Cry1* and *Cry2*) and *Period* (*Per1* and *Per2*) genes to drive their transcription (Figure 5) (van der Horst et al., 1999). E-box elements are basic helix-loop-helix (bHLH) transcription factor-binding sites with the sequence CACGTG. As a consequence, *Cry* and *Per* gene products are synthesized, accumulate in the cytoplasm and together form heterodimeric complexes that move to the nucleus. In the negative limb, when the CRY/PER heterodimer has accumulated to a certain level in the nucleus, CRY/PER inhibits CLOCK/BMAL1-mediated transcription, indirectly shutting *Cry/Per* transcription down. In parallel, *Rev-erbα* (also known as NR1D1 for Nuclear Receptor Subfamily 1, group D, member 1) transcription is activated by the CLOCK/BMAL1 heterodimer and the resulting REV-ERBα/β protein in turn binds the Receptor tyrosine kinase-like Orphan Receptor (ROR)-element in the BMAL1 promoter. In this way, *Bmal1* and other output genes that contain ROR-elements in their promoters are expressed rhythmically in a circadian manner, but in opposite phase from the E-box element-containing genes mentioned above (Preitner et al., 2002).

The most extensive studies on the circadian clock in insects were performed with *D. melanogaster*. In *Drosophila* circadian rhythms are also generated by interlocked transcription/translation feedback loops (Sandrelli et al., 2008). The main loop consists of *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*) and *cycle* (*cyc*) (Stanewsky, 2002). CLOCK and CYCLE form a heterodimer and activate transcription of *per* and *tim* by binding the E-box region (CACGTG) in their promoters. PER and TIM accumulate and dimerize in the cytoplasm and after they translocate to the nucleus, they block the binding of CLK/CYC to DNA and remove CLK/CYC from E-boxes, and as such interfere with transcription driven by CLK/CYC. In *Drosophila*, the internal clock is synchronized to the 24 h cycle by the blue-light photoreceptor CRYPTOCHROME (CRY) (Dubruille and Emery, 2008). CRY is activated by light and binds TIM in order to target TIM for degradation. Additionally, CRY acts as sustainer of peripheral clocks, plausibly by being a transcriptional repressor. It was shown that in a *Drosophila cry* mutant the central circadian oscillator is intact whereas the peripheral circadian oscillators are arrhythmic. As peripheral oscillators are only indirectly light-responsive (via the central oscillator), this demonstrates a photoreceptor-independent role of CRY in the periphery (Krishnan et al., 2001; Collins et al., 2006).

The research on the molecular mechanism of the clock in *Drosophila* encouraged researchers to study other insects revealing differences between *Drosophila* and several

non-dipteran insect species (Sandrelli et al., 2008; Tomioka and Matsumoto 2010). One of the differences is the presence of CRY2 in some insects (e.g. in the monarch butterfly *Danaus plexippus* (order Lepidoptera) and the honey bee *Apis merifella* (order Hymeoptera). Insect CRY2 resembles a mammalian CRY protein that enters the nucleus and acts as a transcriptional repressor of CLK/CYC (Zhu et al., 2008; Rubin et al., 2006). Phylogenetic analysis strongly suggested that due to gene duplication and loss three types of *cry* expression profiles exist in insects: (i) either *cry1* (*Drosophila* type *cry*), or (ii) *cry2* (mammalian type *cry*) or (iii) both *cry1* and *cry2* are expressed (Figure 5) (Yuan et al., 2007; Tomioka and Matsumoto 2010; reviewed by Chaves et al., 2011).

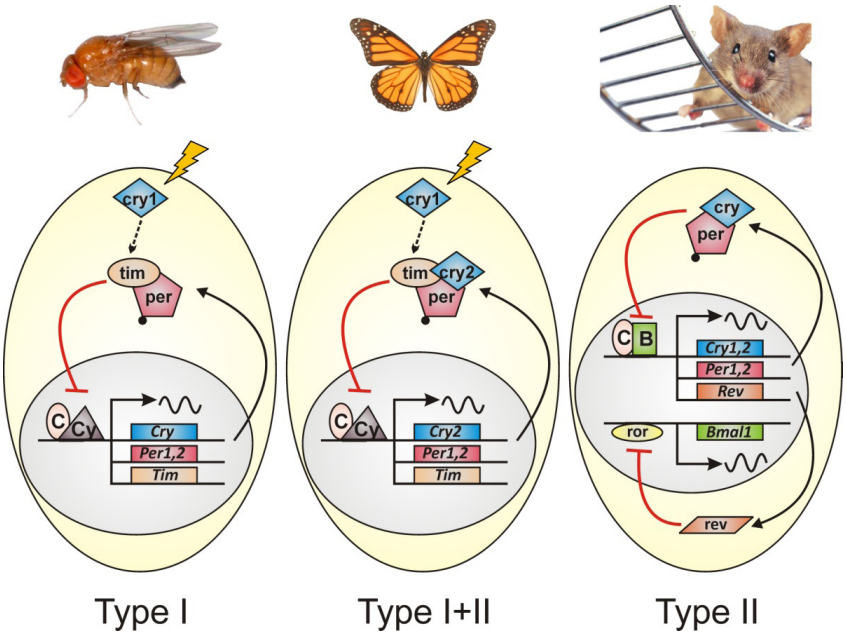

Figure 5. Simplified representation of three molecular oscillators, based on their cryptochrome composition: (*left*) Type I (e.g. fruit fly), (*center*) Type I + II (e.g. monarch butterfly), and (*right*) Type II (mouse). Red lines indicate inhibition, and black dashed arrows indicate light-induced degradation of timeless by interaction with cry. Abbreviations: C, cryptochrome; P, period; T, timeless; Cl, clock; B, Bmal1; Cy, cycle (Chaves et al., 2011).

Scope of the thesis

CPD photolyases are encoded by several baculoviruses and may have the potential to repair UV-induced lesions in DNA in a blue light-dependent manner. Phylogenetic analysis has shown that baculovirus *phr* genes form a single clade indicative of a common ancestor, but the origin of the baculoviral *phr* genes remains enigmatic. It is not known for instance whether a direct ancestral relationship exists between baculoviral and insect *phr* genes. The ChchNPV *phr2* gene (*Cc-phr2*) has been shown to be able to display a DNA repair function in two different systems, i.e. in complementing a deletion in *E. coli* and in repairing T-T dimers *in vitro*. However, a gap remains in our comprehension of the function of the *Cc*I-*phr1* gene in baculovirus biology. It is not clear, for example, whether the potential photoreactivating ability of these photolyases provides the baculovirus

with any ecological benefits and whether the UV repair function is the only function that CPD photolyases have in baculovirus insect-host pathosystems. The aim of this thesis is to address this lack of knowledge by further investigating the function and ecological importance of the CPD photolyases from ChchNPV.

In **Chapter 2** the aim is to shed light on the origin of baculovirus photolyases by analysing the relationship between baculoviral and insect photolyase genes. In order to trace the possible evolutionary path of baculovirus *phr* genes, cDNA sequences are obtained and characterized from the plusiine insects *C. chalcites* and *T. ni*, and from *S. exigua,* and in-depth phylogenetic analysis is performed.

Cc-PHR2, but not Cc-PHR1, has been shown to have photoreactivating abilities in heterologous systems, but it is not known whether these proteins are involved in protection of ChchNPV against UV light. Therefore, a bioassay is developed in **Chapter 3** to determine the mortality caused by UV irradiated ChchNPV OBs in *C. chalcites* larvae. This method is subsequently used to measure the sensitivity of ChchNPV OBs to UV light with or without photoreactivation after UV treatment.

The photoreactivation function may not be the only one displayed by baculovirus photolyases, especially considering the inactivity of Cc-PHR1 in complementation assays. Based on the structural homology to cryptochromes, it is hypothesized that (baculovirus) CPD photolyases might possess an additional function in the circadian clock, similar to the repressive function of cryptochromes. In **Chapter 4** the aim is, therefore, to analyse whether class II CPD photolyases can have a regulatory role in the circadian clock and this is tested for the two ChchNPV PHR proteins. In this context, experiments are performed to determine whether the photolyases can physically interact with components of the molecular oscillator and whether they function to regulate the circadian clock. In **Chapter 5** the PHR protein of marsupial *P. tridactylus (Pt*CPDPL) is tested in the same context as the Cc-PHRs. Additionally, the ability of *Pt*CPDPL to functionally replace mammalian cryptochromes is tested.

In **Chapter 6** a more in-depth analysis of Cc-PHR2 is performed to investigate whether that CPD photolyases can restore rhythmicity of a *cry*-less mutant mammalian oscillator, both in cell culture as in mice. The molecular mechanism that could be used by Cc-PHR2 and *Pt*CPDPL to interact with the molecular oscillator is further investigated.

The general discussion **(Chapter 7)** is dedicated to the question what the function of the baculovirus photolyase is or may be. Furthermore, an outlook will be given to future research.

CHAPTER 2

Baculovirus cyclobutane pyrimidine dimer photolyases show a close relationship with lepidopteran host homologues

Abstract
Cyclobutane pyrimidine dimer (CPD) photolyases repair UV-induced DNA damage using blue-light. To get insight in the origin of baculovirus CPD photolyase (*phr*) genes, homologues in the lepidopteran insects *Chrysodeixis chalcites*, *Spodoptera exigua* and *Trichoplusia ni* were identified and characterized. Lepidopteran and baculovirus *phr* genes each form a monophyletic group, and together form a well-supported clade within the insect photolyases. This suggests that baculoviruses obtained their phr genes from an ancestral lepidopteran insect host. A likely evolutionary scenario is that a granulovirus, *Spodoptera litura* GV or a direct ancestor, obtained a *phr* gene. Subsequently, it was horizontally transferred from this granulovirus to several group II nucleopolyhedroviruses (NPVs), including those that infect noctuids of the Plusiinae subfamily.

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Introduction

DNA damage caused by UV irradiation mainly consists of two types of lesions: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4)-pyrimidone photoproducts (6-4 PPs) (Sancar, 1994). Many organisms, except placental mammals (Yasui et al., 1994), use a repair mechanism based on the action of single enzymes, either CPD photolyases or (6-4) PP photolyases. These enzymes use blue light to restore CPDs or 6-4 PPs, respectively, into their monomeric forms in a PD photolyases. Several chordopoxviruses, (namely the sequenced members of the genera *Avipoxvirus* and *Leporipoxvirus*) and the two sequenced entomopoxviruses (*e.g.*, Bawden et al., 2000) and several baculoviruses encode class II CPD photolyases. Other sequenced poxviruses were not found to possess a *phr* gene.

Baculovirus class II CPD photolyase (*phr)* genes were first detected in *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) and *Trichoplusia ni* (Tn) single nucleocapsid (S) NPV (van Oers et al., 2005; Willis et al., 2005). Later, it was found that all tested alphabaculovirus group II NPVs (Herniou and Jehle, 2007) that infect lepidopteran insect species in the Plusiinae subfamily of the family Noctuidae carry one or more *phr* genes (Xu et al., 2008). In contrast, Plusiinae-infecting group I NPVs, such as *Thysanoplusia orichalcea* (Thor) NPV isolate A28-1, *Rachiplusia ou* (Ro) multiple nucleocapsid (M) NPV and *Anagrapha falcifera* (Anfa) NPV, do not have a *phr* gene (Xu et al., 2008; Harrison and Bonning, 2003).

ChchNPV possesses two *phr* genes named *Cc-phr1* and *Cc-phr2*, which share 45% identity at the amino acid level (van Oers et al., 2005). The *Cc-phr2* gene product can complement an *Escherichia coli* photolyase deficiency and can repair T-T dimers *in vitro*, showing that the Cc-PHR2 protein has photolyase activity (van Oers et al., 2008). The Cc-PHR1 protein did not show photoreactivation activity in these studies. Recently, a *phr* gene was also found in the betabaculovirus *Spodoptera litura* granulovirus (SpliGV) (Wang et al., 2008). *S. litura* is a member of the noctuid subfamily Hadeninae.

Phylogenetic analysis has shown that the above mentioned baculovirus *phr* genes form a single clade indicative of a common ancestor (Xu et al., 2008), but the origin of the baculoviral *phr* genes has not been investigated. Phylogenetically, the baculovirus *phr* genes found so far appeared to group with (entomo)poxvirus homologues (van Oers et al., 2005; Nalcacioglu et al., 2010). This suggests that a *phr* gene could have been introduced into an ancestral baculovirus genome during a concurrent entomopoxvirus infection of a noctuid. Alternatively, a *phr* gene may have been transferred from a plusiine host into the genome of an ancestral plusiine-infecting baculovirus. Since all viruses are parasitic on cellular organisms, it is logical to hypothesize that baculoviruses obtained genetic traits, such as *phr* genes, from their hosts. Other examples of insect host genes probably introduced into baculovirus genomes by horizontal gene transfer are ecdysteroid UDP glycosyltransferase (*egt*) and inhibitors of apoptosis (*iap*) genes (Hughes and Friedman, 2003).

Class II CPD DNA photolyase homologues are known from dipteran insects such as *Drosophila melanogaster* (Yasui et al., 1994) and *Aedes aegypti* (Kim et al., 1996) as well as from the lepidopteran silkworm *Bombyx mori* (Silkbase: silkworm.genomics.org.cn). With this very limited sequence information for lepidopteran *phr* sequences it is impossible to favor one of the above mentioned hypotheses. In order to trace the possible evolutionary path of baculovirus photolyases we characterized *phr* sequences

derived from the plusiine insects *C. chalcites* and *T. ni,* and from *S. exigua*, which belongs to the same subfamily as *S. litura* (subfamily Hadeninae). The latter being the host of the *phr* containing SpliGV. In-depth phylogenetic analysis was performed to analyse the possible relationship between baculoviral and insect *phr* genes.

Materials and Methods

RNA isolation, Reverse Transcription and PCR

Second instars of *C. chalcites*, *S. exigua* and *T. ni* obtained from laboratory cultures in Pamplona (Spain), Wageningen (the Netherlands) and Oxford (United Kingdom), were macerated in 500 µl Trizol (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated following the manufacturers instructions. The RNA pellet was dissolved in 50 µl water and heated for 10 min at 55°C. For RT-PCR analysis, 1 µg of total RNA was reverse transcribed in a total reaction volume of 20 µl. To this aim, the RNA samples were heated at 65°C for 5 min in the presence of 12.5 µM oligo-dT primer (Invitrogen) and 10 mM dNTPs. After cooling on ice, the samples were mixed with 5× first strand buffer (Invitrogen), 10 units of RNAsin (Invitrogen) and 0.2 M DTT, and incubated for 2 min at 37°C. Ten units of SuperScript III Reverse Transcriptase (Invitrogen) were added. The samples were further incubated at 37°C for 70 minutes and finally heated at 70°C for 15 min. The cDNAs obtained were amplified by PCR using photolyase specific degenerate primers PHR-DEG_F and PHR-DEG_R directed against regions with a high degree of conservation at the amino acid level among class II CPD photolyases (encoding QVDAHN and YWAKKI, respectively) as described by Xu et al. (2008). The nucleotide sequences of the PCR products were determined by automated sequencing (Eurofins, Ebersberg, Germany) with universal M13 (-20) F and M13 R primers annealing to 5 extensions of the degenerate PHR primers.

Phylogenetic analyses

CPD photolyase nucleotide sequences were obtained from GenBank (Table 1). BLAST (National Center for Biotechnology Information) was used for nucleotide and predicted amino acid sequence homology searches. Multiple searches were performed, using different baculovirus and lepidopteran photolyases as a query. Sequences from the top hits were included in the analysis, including all known baculovirus (13) and poxvirus (6) *phr* genes, and 15 insect and 7 vertebrate *phr* genes. Sequences were translated in frame to proteins, and protein sequences were aligned using MAFFT version 6 with default settings (Katoh *et al.,* 2005). Protein alignment was converted back into the corresponding codon alignment using PAL2NAL (Suyama et al., 2006). PAUP* version 4.0b10 (Swofford, 2002) was used to select the optimal evolution model, as described in Ros *et al.* (2009). Maximum Likelihood (ML) analysis (heuristic search, 100 bootstrap replicates) was performed in PAUP, using a submodel of the using a submodel of the General Time Reversible Model with invariable sites and a gamma distribution of rate heterogeneity (GTR+I+G) with rate class 'abcade'. (Bayesian inference was conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), using the GTR+I+G model (default settings, 6 million generations, burn-in of 25%).

A *polh/gran* phylogeny was also constructed as described above (model: GTR+I+G, rate class: 'abcadc') and includes the polyhedrin (for NPVs) or granulin (for GVs) sequences of all baculoviruses that have a *phr* gene (except for AmruNPV for

which the polyhedrin sequence is not available in GenBank) and of all other Alpha- and Betabaculoviruses for which the genome sequence is available (Table 2). For the latter ones, the absence of *phr* genes was confirmed (GenBank). Polyhedrin sequences of *Autographa californica* (Ac) MNPV and *Plutella xylostella* (Plxy) MNPV were excluded since they have a mosaic sequence structure (Herniou and Jehle, 2007).

Table 1. Photolyase sequences used for phylogenetic analysis.

Group (Phylum)	Virus family/ Animal order	Species name	GenBank Accession
Virus	*Baculoviridae*	*Apocheima cinerarium* NPV (ApciNPV)	FJ914221*
	Baculoviridae	*Ampelophaga rubiginosa* NPV (AmruNPV)	EU391170
	Baculoviridae	*Clanis bilineata* NPV (ClbiNPV)	DQ504428*
	Baculoviridae	*Chrysodeixis chalcites* NPV (ChchNPV) (NL-phr1)	EU401910
	Baculoviridae	*Chrysodeixis chalcites* NPV (ChchNPV) (NL-phr2)	EU401911
	Baculoviridae	*Pseudoplusia includens* NPV (PsinNPV) (GT-phr1)	EU401912
	Baculoviridae	*Pseudoplusia includens* NPV (PsinNPV) (GT-phr2)	EU682272
	Baculoviridae	*Pseudoplusia includens* NPV (PsinNPV) (LA)	EU401913
	Baculoviridae	*Plusia acuta* NPV (PlacNPV) (GE)	EU401914
	Baculoviridae	*Plusia acuta* NPV (PlacNPV) (USA)	EU682273
	Baculoviridae	*Thysanoplusia orichalcea* NPV (ThorNPV) (B9)	EU401909
	Baculoviridae	*Trichoplusia ni* SNPV (TnSNPV)	DQ017380
	Baculoviridae	*Spodoptera litura* GV (SpliGV)	DQ288858
	Poxviridae	*Amsacta moorei entomopoxvirus* (AMEV)	AF250284
	Poxviridae	*Canarypox virus* (CNPV)	NC_005309
	Poxviridae	*Fowlpox virus* (FPV)	NC_002188
	Poxviridae	*Melanoplus sanguinipes entomopoxvirus* (MSEV)	AF063866
	Poxviridae	*Myxoma virus* (MYXV)	AF170726
	Poxviridae	*Shope fibroma virus* (SFV)	AF170722
Vertebrates	Anura	*Xenopus laevis*	BC133744
(Chordata)	Beloniformes	*Oryzias latipes*	NM_001104801
	Cypriniformes	*Carassius auratus*	D11391
	Cypriniformes	*Danio rerio*	BC054710
	Didelphimorphia	*Monodelphis domestica*	NM_001032977
	Diprotodontia	*Potorous tridactylus*	D26020
	Galliformes	*Gallus gallus*	XM_422729
Invertebrates	Diptera	*Aedes aegypti*	XM_001653905
(Arthropoda)	Diptera	*Anopheles gambiae*	XM_313925
	Diptera	*Culex quinquefasciatus*	XM_001845605
	Diptera	*Drosophila erecta*	XM_001970530
	Diptera	*Drosophila grimshawi*	XM_001986282
	Diptera	*Drosophila melanogaster*	S73530
	Diptera	*Drosophila sechellia*	XM_002032778
	Diptera	*Drosophila virilis*	XM_002059327
	Diptera	*Drosophila willistoni*	XM_002063506
	Diptera	*Drosophila yakuba*	XM_002089497
	Diptera	*Glossina morsitans*	EZ422583
	Hemiptera	*Acyrthosiphon pisum*	XM_001949116
	Hemiptera	*Bemisia tabaci*	EZ962217
	Hymenoptera	*Apis mellifera*	XR_015091
	Hymenoptera	*Nasonia vitripennis*	XM_001603235
	Lepidoptera	*Bombyx mori*	BGIBMGA003618-TA†
	Lepidoptera	*Chrysodeixis chalcites*	**HM044852**
	Lepidoptera	*Spodoptera exigua*	**HM044851**
	Lepidoptera	*Trichoplusia ni*	**HM044850**

Sequences obtained in this study are in bold.
*This sequence was listed as two *phrs* in the genome sequence. For the analysis, the two parts were fused, omitting one frameshift.
†Derived from silkworm.genomics.org.cn

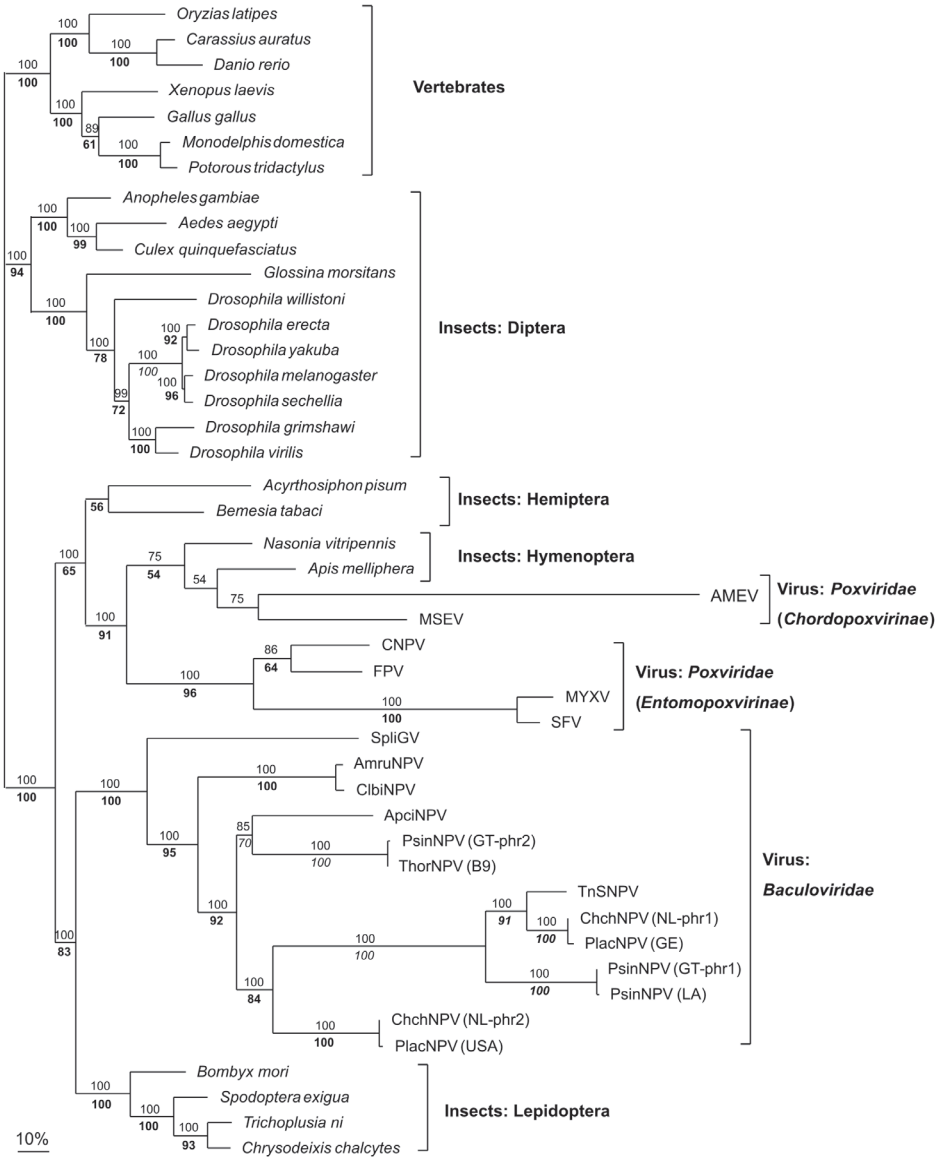

Figure 1. Bayesian phylogeny of photolyase (*phr*) genes from diverse taxa. Numbers in bold indicate Maximum Likelihood bootstrap values based on 100 replicates, plain numbers depict Bayesian posterior probabilities. Only values larger than 50 are indicated for both analyses. The bar at the bottom indicates a branch length of 10% distance. GenBank accession numbers are given in Table 1.

Table 2. *Polyhedrin* and *granulin* sequences used for phylogenetic analysis.

Virus name	Abbreviation	Genbank Accession	Lepidopteran host family name
Adoxophyes honmai NPV	AdhoNPV	AP006270	Tortricidae
Adoxophyes orana GV	AdorGV	AF547984	Tortricidae
Adoxophyes orana NPV	AdorNPV	EU591746	Tortricidae
Agrotis ipsilon MNPV	AgipMNPV	EU839994	Noctuidae
Agrotis segetum GV	AgseGV	AY522332	Noctuidae
Agrotis segetum NPV	AgseNPV	DQ123841	Noctuidae
Antheraea pernyi NPV	AnpeNPV	DQ486030	Saturniidae
Anticarsia gemmatalis NPV	AgNPV	DQ813662	Noctuidae
Apocheima cinerarius NPV	ApciNPV	FJ914221	Geometridae
Bombyx mandarina NPV	BomaNPV	FJ882854	Bombycidae
Bombyx mori NPV	BmNPV	L33180	Bombycidae
Choristoneura fumiferana DEF MNPV	CfDEFNPV	AY327402	Tortricidae
Choristoneura fumiferana MNPV	CfMNPV	AF512031	Tortricidae
Choristoneura occidentalis GV	ChocGV	DQ333351	Tortricidae
Chrysodeixis chalcites NPV	ChchNPV	AY864330	Noctuidae
Clanis bilineata NPV	ClbiNPV	DQ504428	Sphingidae
Cryptophlebia leucotreta GV	CrleGV	AY229987	Tortricidae
Cydia pomonella GV	CpGV	U53466	Tortricidae
Ecotropis obliqua NPV	EcobNPV	DQ837165	Geometridae
Epiphyas postvittana NPV	EppoNPV	AY043265	Tortricidae
Euproctis pseudoconspersa NPV	EupsNPV	FJ227128	Lymantriidae
Helicoverpa armigera GV	HearGV	EU255577	Noctuidae
Helicoverpa armigera MNPV	HearMNPV	EU730893	Noctuidae
Helicoverpa armigera NPV	HearNPV	AF271059	Noctuidae
Helicoverpa zea SNPV	HzSNPV	AF334030	Noctuidae
Hyphantria cunea NPV	HycuNPV	AP009046	Arctiidae
Leucania separata NPV	LeseNPV	AY394490	Noctuidae
Lymantria dispar MNPV	LdMNPV	AF081810	Lymantriidae
Lymantria xylina MNPV	LyxyMNPV	GQ202541	Lymantriidae
Mamestra configurata NPV-A	MacoNPV-A	U59461	Noctuidae
Mamestra configurata NPV-B	MacoNPV-B	AY126275	Noctuidae
Maruca vitrata MNPV	MaviNPV	EF125867	Crambidae
Orgyia leucostigma NPV	OrleNPV	EU309041	Lymantriidae
Orgyia pseudotugata MNPV	OpMNPV	U75930	Lymantriidae
Phthorimaea operculella GV	PhopGV	AF499596	Gelechiidae
Pieris rapae GV	PiraGV	GQ884143	Pieridae
Plusia acuta NPV	PlacNPV	AY706712	Noctuidae
Plutella xylostella GV	PlxyGV	AF270937	Plutellidae
Pseudaletia unipuncta GV	PsunGV	EU678671	Noctuidae
Pseudoplusia includens NPV (GT)	PsinNPV (GT)	EU401916	Noctuidae
Pseudoplusia includens NPV (LA)	PsinNPV (LA)	EU401917	Noctuidae
Rachiplusia ou MNPV	RoMNPV	AY145471	Noctuidae
Spodoptera exigua MNPV	SeMNPV	AF169823	Noctuidae
Spodoptera frugiperda MNPV	SfMNPV	EF035042	Noctuidae
Spodoptera litura GV	SpliGV	DQ288858	Noctuidae
Spodoptera litura NPV	SpliNPV	AF325155	Noctuidae
Spodoptera litura NPV II	SpliNPV II	EU780426	Noctuidae
Thysanoplusia orichalcea NPV	ThorNPV (A28)	AF169480	Noctuidae
Thysanoplusia orichalcea NPV	ThorNPV (B9)	Courtesy of E. Herniou	Noctuidae
Trichoplusia ni SNPV	TnSNPV	DQ017380	Noctuidae
Xestia c-nigrum GV	XecnGV	AF162221	Noctuidae

Results and Discussion

With a set of degenerate primers designed by Xu *et al.*, 2008, partial cDNAs were generated of the *phr* genes of *C. chalcites*, *S. exigua, and T. ni*, following an RT-PCR approach. The resulting cDNAs, approximately 780 base pairs long, were sequenced. Translated BLAST homology searches confirmed that the amplified products encoded class II CPD photolyases. The *phr* sequences were deposited in GenBank under accession number [HM044850] for *Trichoplusia ni,* [HM044851] for *Spodoptera exigua*, and [HM044852] for *Chrysodeixis chalcites*. The *phr* sequences obtained from these lepidopteran insects, together with the *phr* sequences depicted in Table 1 and selected based on top hits in BLAST analyses, were used for phylogenetic analyses to reveal whether and to what extent photolyases from baculoviruses and lepidopteran insects are related. The *phr* phylogeny was rooted with vertebrate *phr* sequences (Figure 1). In order to determine the origin and the evolution of baculovirus photolyases and facilitate the interpretation of the conducted phylogenetic analyses, a *polyhedrin/granulin* (*polh*/*gran*) phylogeny was constructed for baculoviruses (Figure 2). Although based on a single gene, such a phylogeny represents the major clustering of baculoviruses into GVs, group I NPVs and group II NPVs, and has proven to be useful for clustering closely related viruses (Herniou and Jehle, 2007). *Polh*/*gran* genes are conserved and sequences are available for a large number of baculoviruses. The robustness of the phylogenies was evaluated by Maximum Likelihood bootstrap analysis and analysis of Bayesian posterior probabilities (Figures 1 and 2).

CPD photolyase genes (Figure 1) from the lepidopteran insects *S. exigua*, *C. chalcites*, *T. ni* and *B. mori* cluster together forming a monophyletic group. Baculovirus *phr* genes also form a monophyletic group and are closely related to lepidopteran *phr*s, together forming a well supported clade. The position of this clade within the insect photolyases (Figure 1) supports the hypothesis that a *phr* gene has been transferred from a lepidopteran insect to a baculoviruse. The transfer from lepidopteran insects to baculoviruses probably occurred once, and additional transfers followed between baculoviruses. Since baculoviral and lepidopteran *phr* sequences are not mixed in the phylogeny, multiple independent transfers of *phr* genes from lepidopteran hosts to different baculoviral species/isolates seem unlikely.

The most likely evolutionary scenario implies that SpliGV (or a direct ancestor) was the first baculovirus to obtain a *phr* gene from its lepidopteran host, which was subsequently horizontally transferred to other group II NPVs, including the monophyletic plusiine-infecting group II NPVs (Figure 1 and 2). So far, all plusiine group II NPVs analyzed carry one or more *phr* genes (Xu et al., 2008).

A few other non-plusiine infecting NPVs contain a *phr* gene, as is the case for *Ampelophaga rubiginosa* (Amru) NPV, *Clanis billineata* (Clbi) NPV and *Apocheima cinerarium* (Apci) NPV (Figure 1 and Table 1). A likely scenario could be that the NPVs, that infect members of the Sphingidae (AmruNPV, ClbiNPV), were among the first to obtain a *phr* gene, after which the *phr* gene was transferred directly or indirectly to the ancestor of plusiine-infecting group II NPVs and to ApciNPV, which infects a member in the family Geometridae. The exact routes of gene transfer are difficult to infer but this is the most likely scenario based on currently available data. Based on these evolutionary pathways it would be interesting to see whether other NPVs infecting insects in the Geometridae and Sphingidae also contain *phr* genes. However, not all NPVs infecting

geometrid insects contain a *phr* gene, as *Ectropis obliqua* (Ecob) NPV (group II) lacks such a gene (Genbank accession DQ837165).

An alternative evolutionary scenario could have been that a *phr* gene was transferred from an ancestral lepidopteran insect to an ancestor of all baculoviruses. In this case, after acquisition of the *phr* gene by the baculoviral ancestor, *phr* was further distributed concomitantly with speciation of the baculoviruses. However, this scenario is less likely since many independent gene losses are needed to explain the observed *phr* distribution pattern, since many baculoviruses dispersed over the *polh/gran* tree do not have a *phr* gene (Figure 2).

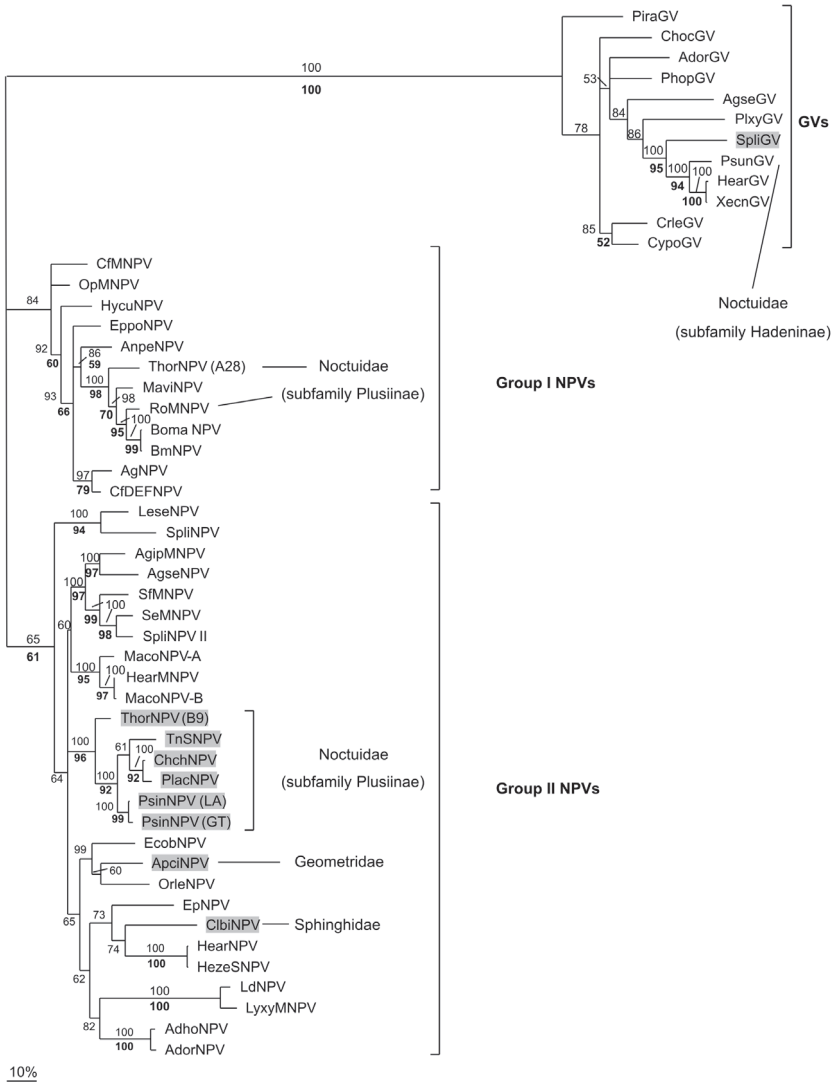

Figure 2. Bayesian phylogeny of baculoviruses based on *polh/gran* genes. Numbers in bold indicate Maximum Likelihood bootstrap values based on 100 replicates, plain numbers depict Bayesian posterior probabilities. Only values larger than 50 are indicated for both analyses. The bar at the bottom indicates a branch length of 10% distance. GenBank accession numbers are given in Table 2. Baculoviruses possessing a phr gene are highlighted in grey. For these viruses, the lepidopteran host family name is depicted.

Another intriguing observation is the presence of two *phr* gene copies within some viral species, such as ChchNPV and the *Pseudoplusia includens* (Psin) NPV isolate from Guatemala (GT). This observation might indicate either a gene duplication event or a horizontal *phr* gene transfer event among baculoviruses or a combination of the two, where a *phr* gene was duplicated in for instance ChchNPV and subsequently one of the copies was transferred to another baculovirus. Duplication events within large dsDNA viral genomes are frequent (Hughes & Friedman, 2005). Alternatively, the baculovirus ancestor might have been equipped with two *phr* gene copies, one of which or both were lost during speciation. Again, this is not a likely scenario since multiple gene losses are needed to explain this scenario.

Interestingly, baculovirus *phr* genes do not form a single clade with those of poxviruses, implying that in contrast to our previous hypothesis (van Oers *et al.*, 2005) *phr* genes have been introduced in viruses in at least two independent events. Baculovirus *phr* genes are closely related to lepidopteran *phr* genes forming a well supported clade, whereas poxvirus *phr* genes are closely related to hemipteran and hymenopteran *phr* genes, also forming a well supported clade (Figure 1). The presence of the two viral groups at different positions within the insect-derived *phr* gene phylogeny suggests that insect *phr* genes are ancestral to viral *phr* genes and implies that baculoviruses and poxviruses independently obtained their *phr* genes from insects, and not from a common viral ancestor (if any) or through horizontal gene transfer between baculoviruses and entomopoxviruses. Poxviruses belong to the cytoplasmic replicating large dsDNA viruses, whereas baculoviruses belong to a group of nuclear replicating large dsDNA viruses with shared features (see the 2009 report of the International Committee on Taxonomy of Viruses (ICTV2009), website: http://www.ictvonline.org/virusTaxonomy.asp?version=2009). The average distance within the virus groups (average ML distance *Baculoviridae*: 1.34, *Poxviridae*: 1.51) is larger than within the insects (Lepidoptera: 0.34, Diptera: 0.60). This could be a consequence of shorter generation times and higher mutation rates of viral genomes.

In the case of the poxviruses, independent transfers from insects to members of the *Entomopoxvirinae* (*Amsacta moorei* entomopoxvirus (AmEV) and *Melanoplus sanguinae* (MsEV) and of the *Chordoxpoxvirinae* (Fowlpoxvirus (FPV), Canarypoxvirus (CnPV), Shope fibromavirus (SFV) and Myxomavirus (MYXV) might have occurred, since the *phr* genes of these two subfamilies do not form a single clade (Figure 1). An alternative scenario is that an ancestor of poxviruses obtained the photolyase from its insect host and now, after speciation, *phr* genes can be detected in both the *Entomopoxvirinae* and *Chordopoxvirinae*. Since the bootstrap support is low within this cluster, it is impossible to infer the exact position of the poxvirus *phr* genes and to make a choice between these two scenarios.

In this paper, we characterized CPD photolyase gene sequences in lepidopteran insects that serve as hosts for baculovirus infections and determined their phylogenetic relationship with other *phr* genes. We conclude that the *phr* genes of lepidopteran insects are highly related to the baculovirus homologues and together form a well supported clade. This strongly supports the hypothesis that a CPD photolyase gene was introduced into the family *Baculoviridae* by a single horizontal gene transfer from an ancestral lepidopteran host into an ancestral baculovirus.

Acknowledgements

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CHAPTER 3

Baculovirus photolyases do not reduce the sensitivity of occlusion bodies to ultraviolet (UV) irradiation

Abstract
The use of baculoviruses in insect biocontrol is hampered by their sensitivity to ultraviolet (UV) light, which induces cyclobutane pyrimidine dimers (CPDs) in DNA. CPD-photolyases repair CPDs using visible light. Whether the photolyases encoded by *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV), Cc-PHR1 and Cc-PHR2, reduce the UV-sensitivity of this virus, was tested by infecting larvae with UV-irradiated viral occlusion bodies (OBs) that were subsequently treated with visible light or kept in the dark. The observed mortality was statistically the same for both treatments. We postulate that Cc-PHRs are not active as DNA repair enzymes in OBs but may be active intracellularly at an early infection stage. Alternatively these PHRs play a different role in baculovirus pathogenesis.

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Introduction

Sunlight is (in)directly the main source of energy for all organisms. At the same time, the ultraviolet (UV) component of sunlight can have destructive effects by causing lesions in DNA: cis-syn-cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6,4)-pyrimidone photoproducts (6-4PPs) (Müller and Carell, 2009). CPDs are formed when two adjacent pyrimidines, usually thymines, are linked by two covalent bonds (Essen and Klar, 2006). To deal with the harmful effects of UV-irradiation, most organisms (except placental mammals) rely on lesion-specific photolyases to repair UV-induced damage in a light-dependent manner (Brettel and Byrdin, 2010). CPD-photolyases need both redox active flavine adenine dinucleotide (FAD) and an antenna molecule as cofactors (Park et al., 1995).

CPD-photolyases are conserved in a specific group of baculoviruses that infect plusiine insects (Xu et al., 2008). Baculoviruses are large, enveloped double-stranded DNA viruses that infect invertebrates, predominantly insects in the orders Lepidoptera, Hymenoptera and Diptera (Slack and Arif, 2007). The first baculovirus CPD-photolyase (*phr*) genes were found in *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) and were named Cc-*phr1* and Cc-*phr2* (van Oers et al., 2004, 2005). They share 45% identity at the amino acid level. Cc-PHR2, in contrast to Cc-PHR1, was shown to possess CPD-photolyase activity in a heterologous system and *in vitro* (van Oers et al., 2008), but there is no experimental evidence that these photolyases repair UV-light-induced DNA damage in ChchNPV occlusion bodies (OBs) prior to infection or at all. Baculoviruses are applied as biocontrol agents since the 1950s as a host-specific alternative to chemical pesticides (Szewczyk et al., 2006), but quick inactivation by UV-light in the field poses a severe constraint on their use (Inceoğlu et al., 2001; Sun and Peng, 2007). To limit UV inactivation expensive UV protectants are added to baculovirus formulations (Black et al., 1997).

To investigate whether the presence of Cc-PHR1 and/or Cc-PHR2 makes ChchNPV less sensitive to UV-light, we UV-irradiated viral OBs to inactivate the DNA in the occlusion derived form of the virus (ODV) and then exposed the OBs to visible light to allow photolyases to repair the CPD lesions. A second group of OBs was kept in the dark after UV-irradiation (no photoreactivation) to serve as a negative control. The sensitivity of ChchNPV to UV-light was determined by comparing the mortality of *C. chalcites* larvae fed with the two groups of UV-irradiated OBs.

Materials and methods

Bioassays and lethal dose establishment

A laboratory colony of the tomato looper, *C. chalcites*, was reared on artificial diet at 28 ± 1 ºC at a 16-h light/8-h dark photoperiod (Murillo et al., 2000). The Dutch isolate of ChchNPV (ChchNPV-NL) was described before (van Oers et al., 2004). The 90% lethal concentration (LC90) of wild-type (wt) ChchNPV-NL was determined in insect bioassays using droplet feeding (Hughes and Wood, 1981), with in total ~75 larvae and five different concentrations of OBs. OBs were isolated by grinding virus-killed cadavers in sterile water, filtering the homogenate through muslin and sedimentation at 6000rpm for 5 min. Virus suspension for droplet feeding was prepared by serially diluting the OB stock solutions with sterile dH_2O. A suspension containing 10% sucrose, 0.001% Fluorella

blue, and 0, 4.4× 10^5, 8.8×10^4, 1.7× 10^4, 3.5×10^3 or 7× 10^2 OBs/ml was given to 2nd instars, which were starved for 24h prior to droplet feeding. A mock infection with dye only served as negative control. Subsequently, larvae were transferred to individual wells of 24-well plates containing diet. Mortality was recorded daily until 8 days post infection (p.i.) and the data were analyzed using Polo Plus (LeOra Software, 1987).

Determination of UV dose

To determine the UV dose needed to inactivate ChchNPV OBs, 0.5ml suspensions of 5×10^6 OBs/ml, were irradiated in 35 mm Petri dishes (Nunc) with 250 nm UV-light at total doses of 0, 50, 100, 150, 200 or 300 J/m^2 measured by a UV-meter (UVP, LLC Upland, CA) The irradiated OB suspension was kept in the dark for 6h. Subsequently, starved *C. chalcites* 2nd instars (25 insects per treatment, three-times repeated) were droplet-fed 5×10^6 OBs/ml and monitored for mortality as described above.

Laboratory bioassays for UV resistance

ChchNPV OBs were irradiated at UV dose of 0 or 200 J/m2. The irradiated samples were either incubated in complete darkness or exposed to visible light with a regular 13W TL-tube (Philips) at 28 ± 1 ºC for 30 min, 1h, 2h or 6h. An 8 mm glass plate was used to filter out short wavelength UV-light. Two independent bioassays were performed as described above.

Results and Discussion

The discovery of CPD-photolyases in baculoviruses (van Oers et al., 2004; Willis et al., 2005) provided a potential novel tool to reduce the UV-sensitivity of baculoviruses used for biocontrol and so to improve the survival of baculoviruses in the field. Cc-PHR1 and Cc-PHR2 were tested, therefore, for DNA repair activity *in vivo*. In the first experiment, the LC90 of ChchNPV for 2nd instar *C. chalcites* larvae was determined as 2.8×10^6 OBs/ml (χ^2=3.14; degree of freedom: 3; heterogeneity: 1.05). A virus concentration high enough to kill approximately 100% of the larvae (5×10^6 OBs/ml) was subsequently used to establish the dose of UV-light that would reduce the mortality to ~10%. When ChchNPV OBs were irradiated with UV-light in an aqueous suspension, and subsequently fed to larvae, mortality was observed in a range: 0-200 J/m^2 (Table 1). The mortalities of *C. chalcites* larvae decreased gradually with increasing UV dose and reached ~12% at 200 J/m^2.

In order to determine whether Cc-PHR1 and/or Cc-PHR2 are active repair enzymes in ChchNPV OBs or early after infection in that they can rescue virus inactivation by UV-light, a bioassay was performed with OBs irradiated with UV dose of 200 J/m^2 (Figure 1). Non-irradiated OBs (0 J/m^2) were used as a positive control and resulted in 100% mortality of *C. chalcites*. No significant difference was found for ChchNPV exposed to visible light (photoreactivation) or kept in the dark after UV-irradiation, irrespective of how long visible light was applied (Figure 1). Hence, the bioassay showed, that the photolyases encoded in the ChchNPV genome are not active in OBs of ChchNPV and do not protect ChchNPV at this stage against UV-irradiation.

It is important to note that in a recent proteomic study both photolyases were not detected in the ChchNPV ODV particles (the virus in the OBs) (Xu et al., 2011). These data combined with the data of the current study indicate that both photolyases are

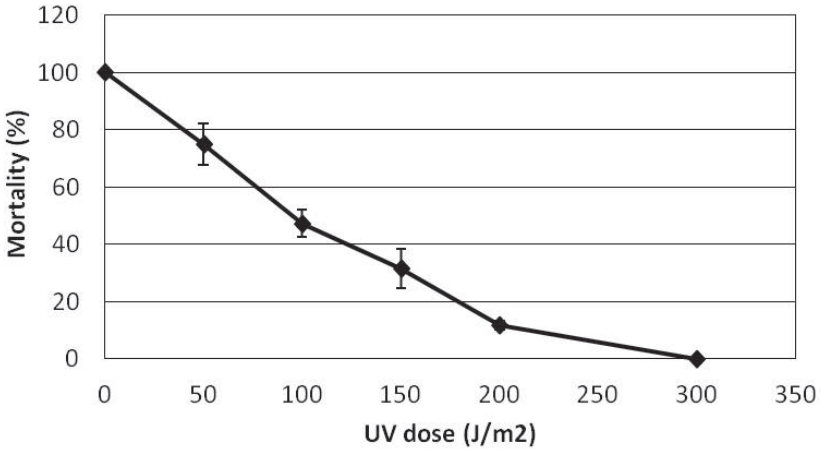

Figure 1. UV-sensitivity of ChchNPV OBs. The mortality (%) of *C. chalcites* larvae infected with OBs from ChchNPV treated with different UV-light doses (J/m^2) was measured. Mean and standard deviation of triplicate samples are shown.

not present in ODVs and hence cannot repair DNA in the ODV particle prior to infection or in the very early stages of infection before gene expression. The fact that these proteins were not found in ODVs, however, is in line with the presence of baculovirus early putative promoter motifs (CAGT for phr1; GATA for phr2) and early gene expression (van Oers et al., 2004; 2005). The most likely scenario thus is that baculovirus photolyases are expressed in the early stage of infection only and are routed to the nucleus, as shown in transient expression assays (Xu et al., 2010). In the latter study, PHR2 was co-localized with the nuclear virogenic stroma, the site of virus replication. However, in a real infection, Cc-PHR2 may repair DNA lesions early in infection or participate in the replication repair (Huang et al., 2011), but is no longer required when ODV particles are assembled. The early expression may be a consequence of the origin of the baculovirus *phr* genes, which are most likely derived from an ancestral insect host (Biernat et al., 2011) and may have been inserted in the viral genome together with an insect promoter, only active during the early phase of baculovirus infection.

The situation for baculovirus photolyases is different, therefore, from what was shown for Fowlpox virus, which appeared to incorporate its photolyase into mature virions, as the enzyme was able to repair UV-induced DNA damage in extracellular virus particles (Srinivasan et al., 2001). Proteomic analysis of the occluded virions of *Amsacta moorei* entomopox virus also showed association of the virus-encoded photolyase with the virion (S. Perera and B. Arif, personal communications).

This leaves open the question, what the function of the baculovirus photolyases could be. It is known that photolyases are homologous to cryptochromes, proteins that function in the circadian clock to regulate oscillation mechanisms, and hence, physiology, behavior and metabolism of almost all organisms (van der Horst et al., 1999). PHR2 can potentially function in the circadian clock by mimicking the role of cryptochrome (Biernat et al., 2012). We postulate, therefore, that PHR2 could have an effect on the circadian clock of the insect host. PHR2 expressed early in infection may, therefore, not only protects against UV-light in this stage of infection, but may also play a role in virus-

induced behavioural changes. Infected larvae become hypermobile and start to climb (Goulson, 1997; Hoover et al., 2011) with a putative benefit for virus transmission as OBs can be easier and more efficiently spread over the foliage. To fully understand the properties of baculovirus photolyases, studies in *Chrysodeixis chalcytes* larvae with *phr* knockout viruses are needed.

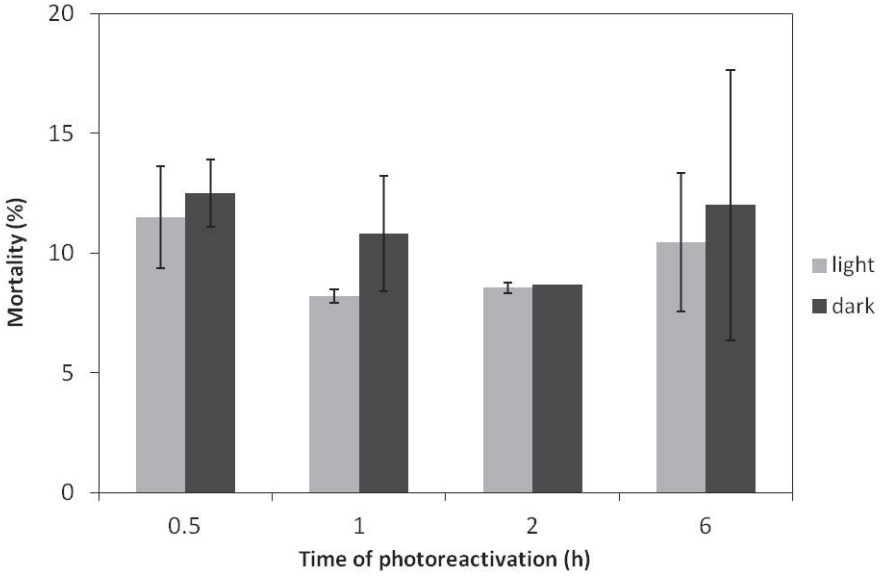

Figure 2. Photoreactivation capacity**.** The mortality (%) of *C. chalcites* larvae was recorded after infection with OBs from ChchNPV previously exposed to visible light or kept in the dark after UV-irradiation at a UV dose of 200 J/m^2. Mean and standard deviation of duplicate samples are shown.

In conclusion, we have shown that there is no difference in UV-sensitivity of ChchNPV with or without photoreactivation and that the PHRs must play a different role in baculovirus pathogenesis and/or behaviour. It is likely that the photolyases are expressed only in the early phases of infection and, at least in the case of Cc-PHR2, may have even a dual function: as an active CPD-photolyase and as a modulator of the circadian clock, with possible consequences for insect metabolism and behavior.

Acknowledgments

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CHAPTER 4

A baculovirus photolyase with DNA repair activity and circadian clock regulatory function

Abstract
The cryptochrome/photolyase gene family (CPF) encodes proteins that exhibit either photoreceptor or DNA repair activity. Cryptochromes and photolyases are structurally conserved, but display distinct functions. Photolyases use visible light to repair UV-induced DNA damage. Cryptochromes, however, function as blue-light receptors, circadian photoreceptors, or repressors of CLOCK/BMAL1 heterodimer, the transcription activator controlling the molecular circadian clock. We have obtained evidence that the functional divergence between cryptochromes and photolyases is not so univocal. *Chrysodeixis chalcites* nucleopolyhedrovirus possesses two photolyase-like genes: *phr1* and *phr2*. We show that PHR1 and PHR2 are able to bind the CLOCK protein. Only for PHR2, however, the physical interaction with CLOCK represses CLOCK/BMAL1-driven transcription. This finding shows that binding of photolyase per se is not sufficient to inhibit the CLOCK/BMAL1 heterodimer. PHR2, furthermore, affects the oscillation of immortalized mouse embryonic fibroblasts, suggesting that PHR2 indeed regulates the molecular circadian clock. These findings might have a high relevance not only for further understanding of the evolution of CPF members, but also for understanding of behavioural changes induced by baculoviruses in their insect hosts.

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Introduction

Photolyases are DNA damage repair enzymes that lesion-specifically and in a light-dependent manner remove cyclobutane pyrimidine dimers (CPD) or pyrimidine-(6,4)-pyrimidone photoproducts ((6-4)PP) from UV-exposed DNA in a process called photoreactivation (reviewed by Müller and Carell, 2009; Eker et al., 2009). Photolyases, together with cryptochromes, belong to the cryptochrome/photolyase family (CPF) of flavoproteins. Cryptochromes were for long known as plant blue-light receptors (Cashmore et al., 1999). More recently homologues of cryptochromes were identified in animals, were they act as combined circadian photoreceptor/core clock proteins (in e.g. *Drosophila* and zebrafish), as pure core clock proteins (in mammals and *Xenopus*), or as magnetoreceptor proteins (in insects and birds) (Kume et al., 1999; van der Horst et al., 1999; Cashmore et al., 2003; Gegear et al., 2010; Ritz et al., 2000). A new class of CPF proteins with CPD photolyase activity for single strand DNA was identified recently. The proteins are termed Cry-DASH (cryptochrome - *Drosophila*, *Arabidopsis*, *Synechocystis*, Human) to indicate that Cry-DASH show higher sequence homology to *Drosophila* and human cryptochromes than to bacterial photolyases (Hitomi et al., 2000; Brudler et al., in 2003; Daiyasu et al., 2004, Pokorny et al., 2008).

Cryptochromes and photolyases share a well conserved core domain of about 500 amino acids (photolyase-like domain) which binds two chromophores: flavin adenine dinucleotide (FAD) and either 5,10-methenyl-tetrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyl-5-deazariboflavin (8-HDF). Cryptochromes lack the nuclear localization signal-containing N-terminal extension characteristic for eukaryotic photolyases. Except for Cry-DASH, cryptochromes are equipped with a C-terminal extension of variable length (Daiyasu et al., 2004; reviewed by Eker et al., 2009). It has been proposed that the presence of different (species-specific) C-terminal extensions functionally separates cryptochromes from photolyases and Cry-DASH underlying the functional diversity (i.e. photoreceptor, magnetoreceptor, and circadian core oscillator function) within the cryptochrome family (Chaves et al., 2006).

The mammalian cryptochromes (*Cry1* and *2*) are indispensable elements of the circadian clock (van der Horst et al., 1999) that allows most, if not all, organisms to anticipate to the environment by adjusting metabolism, physiology and behavior to the momentum of the day. Circadian rhythms are generated by a self-sustained molecular clock, which has an intrinsic periodicity of approximately 24 h, and accordingly needs to be reset daily to keep pace with the day/night cycle as imposed by the rotation of the earth around its axis (Young and Kay, 2001). Although circadian clocks can respond to various cues (e.g. food availability, DNA damage), the main “Zeitgeber” or cue is visible light (Dijk et al., 1995).

The circadian clock is based on an auto-regulatory molecular oscillator in which a set of clock genes is periodically activated and repressed via interlocked positive and negative transcription/translation feedback loops (TTFL) (Reppert and Weaver, 2001). In mammals, the main players in this molecular clockwork are the *Clock*, *Bmal1*, *Rev-Erbα*, *Period* (*Per1* and *2*) and *Cryptochrome* (*Cry1* and *2*) genes. In short, CLOCK and BMAL1 heterodimerize to form a transcription activator that binds to E-box promoter elements (CACGTG) and turns on expression of the *Cry*, *Per* and *Rev-Erbα* genes. Once synthesized, the CRY and PER proteins form complexes that inhibit CLOCK/BMAL1-driven transcription, which also affects *Cry* and *Per* mRNA synthesis (Shearman et al.,

2000). In a second feedback loop REV-ERBα inhibits the receptor tyrosine kinase-like orphan receptor (ROR)-driven transcription of *Bmal1* (Preitner et al., 2002). In addition, posttranslational modification of clock proteins (e.g. phosphorylation, ubiquitylation, acetylation) controls the stability and subcellular localization of the clock proteins, thereby fine-tuning the period length of the clock (Gallego and Virshup, 2007; Vanselow and Kramer, 2007). The circadian core oscillator is coupled to rhythmic output pathways via E-box and RORE containing clock-controlled genes (Reppert and Weaver, 2002; Takahashi et al., 2008).

The common ancestor for CPF members is probably a photolyase that made organisms more resistant against UV-light (Cashmore et al., 1999). The photoreceptor function has developed later to help organisms to sense day-night changes in order to avoid ultraviolet irradiation (reviewed by Gehring and Rosbach, 2003). From phylogenetic analysis it was deduced that animal cryptochromes are more closely related to (6-4)PP photolyases, whereas plant cryptochromes presumable evolved from CPD photolyases. This phylogenetic analysis, therefore, suggests that animal and plant cryptochromes arose by two independent evolutionary events (Cashmore et al., 1999). Although cryptochromes were initially described as limited to eukaryotic organisms, subsequent findings showed that they are also present in bacteria (Hitomi et al., 2000). Further characterization of Cry-DASH pointed out that cryptochromes evolved before the divergence of prokaryotes and eukaryotes (Brudler et al., 2003; Kleine et al., 2003).

Members of CPF can also be encountered in various viruses. Baculovirus CPD photolyase-like genes were first described in *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) and were designated *phr1* and *phr2* (van Oers et al., 2004). PHR2 can rescue bacteria lacking a UV repair system, whereas PHR1 does not (van Oers et al., 2008). Further characterization indicated that the *phr2* gene, in contrast to *phr1*, encodes a photolyase with DNA repair activity towards UV-induced CPDs.

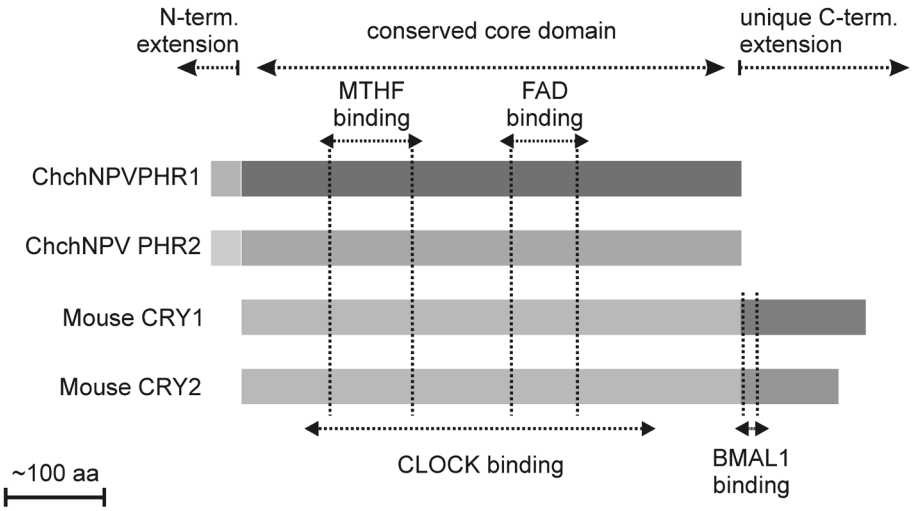

Figure 1. Comparison between PHRs and mammalian CRYs. Schematic representation of *phr1*, *phr2* and mouse CRY1 and 2. Conserved and unique domains are indicated above, chromophore binding sites are indicated by vertical doted lines. CLOCK and BMAL1 binding regions in mouse CRY and indicated below.

The diversity in functions between cryptochromes and photolyases was discussed recently for (6-4)PP photolyases (Coesel et al., 2009; Heijde et al., 2010) and for CPD photolyase (Chaves et al., submitted). The functional diversity and evolution of CPF members, however, is not understood entirely. Except the evolutionary question, duality of function might have an important physiological impact. Insects are known to change their behaviour after baculovirus infection, but underlying mechanisms are still not specified (Goulson, 1997). A possible clock regulatory function of PHR1 and PHR2 would have a high relevance, not only for understanding the evolution of CPF, but also for understanding the baculovirus-induced behavioural changes. We investigated, therefore, whether PHR1 and PHR2 are able to interact with components of the mammalian molecular clock and, consequently, have a transcription regulation function. We studied also the effect of baculoviral photolyases on the molecular oscillator and examined their possible function in the circadian clock.

Material and Methods

Construction of plasmids

Flag-tagged mouse *Bmal1* and *Clock* expression constructs (further referred to as *Bmal1-flag* and *Clock-flag*) and the *HA-mCry1* expression construct (all in pcDNA3) have been described previously (Chaves et al., 2006). To express ChchNPV *phr1* and *phr2* in mammalian cells, the complete coding region of both genes was PCR-amplified from genomic ChChNPV DNA using Phusion high-fidelity DNA polymerase (Finnzymes) and restriction site flanked primers. The obtained *Hin*dIII-*Bam*H1 *phr1* and *Eco*RI-*Bam*HI *phr2* fragments were cloned into pcDNA3.1 (Invitrogen) to give pcDNA3.1-phr1 and pcDNA3.1-phr2. Essentially the same PCR strategy was taken to generate constructs for the expression of C-terminally EGFP-tagged PHR, except that the reverse PCR primers were designed in such a way that the 3' *Bam*HI restriction site replaced the *phr* STOP codon. The obtained *Hin*dIII-*Bam*H1 *phr1* and *Eco*RI-*Bam*HI *phr2* fragments were first cloned into pJET (Fermentas) and subsequently recloned upstream of the *egfp* coding sequence of pEGFPN3 and pEGFPN1 (Clontech), respectively. The pEGFPN3-*phr1* and pEGFPN1-*phr2* constructs are referred to as *phr1-egfp* and *phr2-egfp*.

Cells, culture and transient transfection conditions

HEK 293T (Human Embryonic Kidney) and NIH 3T3 (immortalized mouse embryonic fibroblast) cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)/Ham-F10 medium (1:1; Lonza), supplemented with 10% fetal calf serum (Lonza), penicillin and streptomycin. Transient expression of proteins was achieved by transfecting cells with afore mentioned constructs using FuGene reagent according to the manufacturer's instruction (Roche).

Co-Immunoprecipitation and Western Blot analysis

For co-immunoprecipitation studies, HEK 293T cells transiently (co)expressing *phr1-gfp* or *phr2-gfp*, *Bmal1-flag* and/or *Clock-flag* were lysed 24 h after transfection in immunoprecipitation (IP) buffer, composed of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1mM EDTA, and supplemented with a complete protease inhibitor cocktail (Roche) and phosphatase inhibitors (Pierce) and sonicated. After centrifugation (20 min, 16000xg, 4 ˚C), a small amount of the lysate was stored (input) and the remainder was

incubated with an anti-GFP antibody (Roche) for 3 h at 4 ^{o}C under continuous rotation. After addition of G beads (GE Healthcare), incubation was continued for another hour, after which the beads were washed twice with IP wash buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0, 1% NP-40 and 0,05 % sodium deoxycholate). Next, the beads and input samples were boiled in 2× Laemmli sample buffer and separated by SDS polyacrylamide gel electrophoresis in 4-12% gradient SDS-PAGE gels (Invitrogen). Subsequently, proteins were transferred to a nitrocellulose membrane and visualized using anti-Flag (Sigma; 1:1000 dilution) or anti-GFP (Roche; 1:1000 dilution) primary antibodies. Horseradish peroxidase-conjugated anti-rabbit IgG (Dako; 1:5000 dilution) was used as a secondary antibody and chemoluminescence was detected using ECL Western Blotting Reagents (GE Healthcare).

Immunofluorescence

HEK 293T cells were grown on cover slips in 12-well plates (10^5 cells per well) and (co) transfected with *phr1-gfp*, *phr2* and/or *flag-clock* using Fugene reagent (Roche). After 24 h cells were fixed for 10 min with 4% paraformaldehyde in PBS. After washing 3 times with PBS, cells were permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min and washed with PBS. Subsequently, cells were blocked for 10 min in PBS containing 1.5 % BSA and 100 mM glycine, and incubated at room temperature with mouse anti-FLAG (Sigma; 1:1000 dilution), mouse anti-GFP (Roche; 1:1000 dilution) or rabbit anti-PHR2 (Xu et al., 2010; 1:500 dilution) antibody was performed. As a secondary fluorescent anti-rabbit antibody-Alexa 488 (Invitrogen; 1:1000 dilution) or anti-mouse antibody-Alexa 594 (Invitrogen; 1:1000 dilution) were used. Fluorescence images were taken with a Leica DM-RB microscope.

Luciferase reporter assay

The CLOCK/BMAL1 transcription assay was performed as previously described (Yamaguchi et al., 2000). NIH 3T3 cells were seeded in 24 well plates (5×10^4 cells per well) and cotransfected with the *mPer2::luciferase* reporter construct (1 ng), pRL-CMV null-*Renilla luciferase* (0.01 ng; used as an internal control), *Clock* (100 ng)*, Bmal1* (100 ng) *,* and either *mCry1* (100 ng, used as a positive control for inhibition of CLOCK/BMAL1 transcription), pcDNA3.1-*phr1* (200 ng) or pcDNA3.1-*phr2* (200 ng) expression construct, using Fugene (Roche). The total amount of 1,2 µg DNA per transfection was adjusted by adding pcDNA3 vector. Each transfection was performed in triplicate. After 24 h transcriptional activity was assessed with the Dual-Luciferase 10 Reporter Assay (Promega) by measuring the ratio of firefly activity to *Renilla* luciferase activity. For statistical analysis, a two-tailed *t*-test was used.

Real time luminometry

For real-time bioluminescence monitoring of circadian core oscillator performance, NIH 3T3 cells were cotransfected with the *mPer2::luciferase* reporter construct and either pcDNA3.1-phr1, pcDNA3.1-phr1-egfp, or pcDNA3.1-phr2. As a control, we used pcDNA3.1 (empty vector) or pEGFP. Two days after transfection, cells were clock-synchronized by replacing the medium with 2 ml fresh medium containing forskolin (30 µM), luciferin (100 µM), HEPES (25 nM), 10 % fetal calf serum and antibiotics. Dishes were sealed with a glass coverslip and parafilm and placed in a LumiCycle 32-channel automated luminometer (Actimetrics) in a dry, temperature-controlled incubator at

37 °C. In a parallel series of experiments, the *Bmal1::luciferase reporter* was used. Real time bioluminescence recording (60 sec measurements at 10 min intervals) and data processing have been described before (Oklejewicz et al., 2008).

Results

PHR1 and PHR2 can physically interact with CLOCK

Taking into account the structural similarity between cryptochromes and photolyases as well as the recent observation that *Potorous tridactylus* CPD photolyase can replace CRY proteins in the repression of CLOCK/BMAL1 driven transcription (Chaves et al., submitted), the first step in studying whether baculovirus photolyases may potentially exhibit a circadian clock function was to determine whether PHR1 and/or PHR2 can physically interact with CLOCK and BMAL1, the positive elements of the mammalian transcription/translation feedback loop. To this end we performed pull-down experiments using HEK 293 cells transiently expressing *phr1-egfp* or *phr2-egfp* in combination with either *Flag-Clock* or *Flag-Bmal1.* As shown in Figure 1A, PHR1 failed to co-precipitate with BMAL1 whereas, interestingly, the protein could be pulled down together with the CLOCK protein. In the absence of the CLOCK protein, PHR1 could not be precipitated with anti-FLAG antibodies, showing that the co-precipitation of PHR1 is not an aspecific feature of the antibody used. Similar results were obtained for PHR2 (Figure 1B). From these data we conclude that the PHR1 and PHR2 proteins can physically interact with CLOCK.

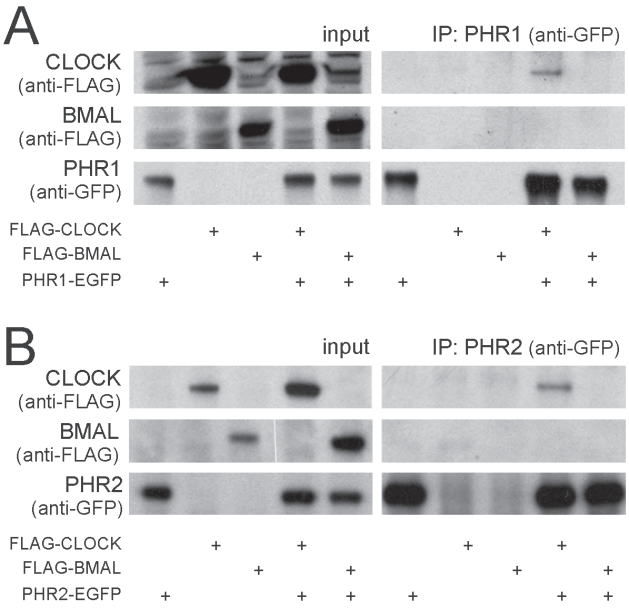

Figure 2. PHR1-EGFP and PHR2-EGFP interact with FLAG-CLOCK. HEK 293T cells were transiently transfected with *phr1-egfp* (A) or *phr2-egfp* (B), Flag-Clock, Flag-Bmal1 and combinations of phr1-egfp or phr2-egfp with Flag-Clock or Flag-Bmal1, followed by Western blot analysis. The presence of FLAG-tagged CLOCK and BMAL1 was detected with anti-FLAG antibody. The presence of PHR1-EGFP and PHR2-EGFP was confirmed using anti-GFP antibody. FLAG-CLOCK is clearly visible after co-immunoprecipitation of PHR1-EGFP and PHR2-EGFP proteins with anti-GFP antibody, but FLAG-CLOCK is absent when there is no photolyase expressed in the sample (A and B, IP panel).

PHR1 and PHR2 co-localize with CLOCK

Overexpressed FLAG-CLOCK mainly localizes in the cytoplasm but, when co-expressed with CRY1, it completely accumulates in the nucleus (Chaves et al., 2006). To investigate the consequences of the interaction between CLOCK and the PHR proteins on the subcellular localization of CLOCK, we performed an immunofluorescence assay in HEK 293T cells transiently expressing these proteins (Figure 2A and 2B). As might be expected

on the basis of the predominantly nuclear localization of baculoviral photolyases in insect Sf9 cells and their strong affinity to condensed DNA (Xu et al., 2010), PHR1 and PHR2 were also found mainly in the nucleus (Figure 2A). When CLOCK was co-expressed with PHR1 or PHR2, the subcellular localization of CLOCK shifted from cytoplasmic to nuclear (Figure 2B). This finding not only further illustrates that baculoviral PHR proteins can interact with CLOCK, but also suggest that they may affect the performance of the circadian core oscillator.

PHR2 acts as an inhibitor of CLOCK/BMAL1-driven transcription

In order to test whether the interaction of PHR1 and/or PHR2 with CLOCK affects the transcription activation properties of the CLOCK/BMAL1 heterodimer, we performed an *mPer2::luciferase* reporter gene-based transcription inhibition assay in NIH 3T3 cells. The co-expression of the reporter gene with Clock and Bmal1 caused an increase in the transcription of the reporter gene compared to mPer2::luciferase alone (Figure 3). As expected, co-expression of mCRY1 drastically inhibited CLOCK/BMAL1-driven transcription. When mCRY1 was replaced by PHR1 or PHR2, only the latter protein was capable of consistently and significantly ($p<0.01$) reducing the transcriptional activity of the CLOCK/BMAL1 heterodimer (Figure 3). This finding suggests that despite the binding of PHR1 to CLOCK, this intereaction is not sufficient to inhibit of CLOCK/BMAL1.

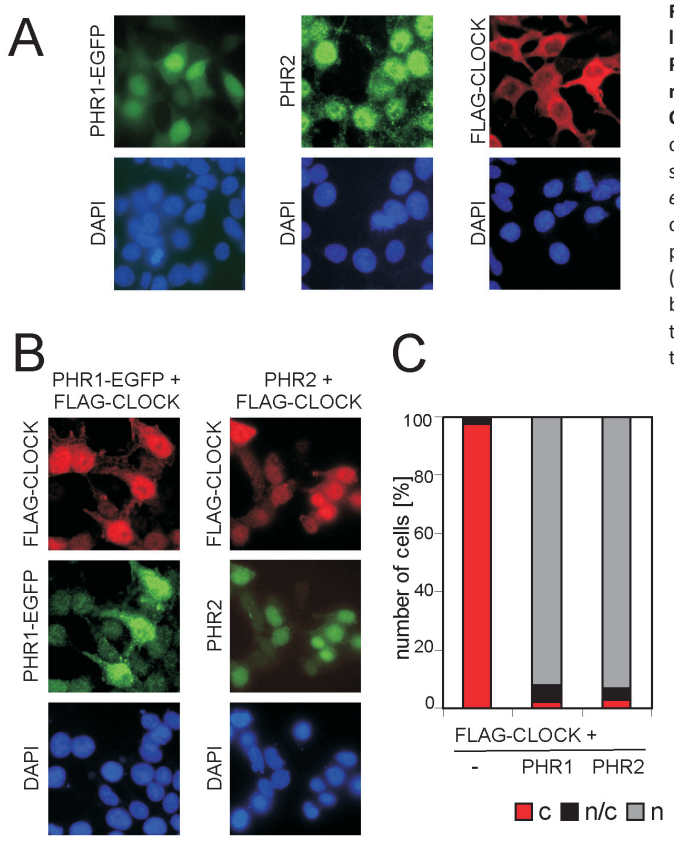

Figure 3. Subcellular localization of PHR1-EGFP, PHR2, FLAG-CLOCK and nuclear transport of FLAG-CLOCK. Immunofluorescence of HEK 293T cells transiently single transfected with *phr1-egfp*, *phr2*, *Flag-Clock* (A) or with combinations of photolyase and *Flag-Clock* (B). DAPI nuclear staining in blue. (C) Quantification of the cellular localization of the transiently expressed proteins.

PHR2 dampens the oscillation of synchronized NIH3T3 cells

To obtain insight into the physiological consequences of overexpression of PHR1 or PHR2 on the molecular clock, we next investigated the core oscillator performance in forskolin-synchronized NIH 3T3 cells transiently co-expressing the *Bmal1::Luc* reporter plasmid (Figure 4). This was done in combination with either *phr1-egfp* or *phr2* expression

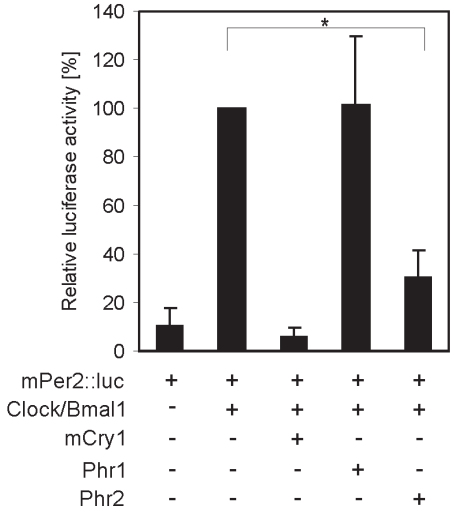

Figure 4. Relative luciferase activity in NIH 3T3 cells. Relative luciferase activity in NIH 3T3 cells transiently transfected with Per2::luciferase construct alone or in combination with various plasmids as indicated below the graph. The luciferase activity in samples that contained *Clock/Bmal1* was taken as 100%. Error bars (±s.e.) were calculated from three independent experiments. p-values lower than 0.05 are indicated with asterixes.

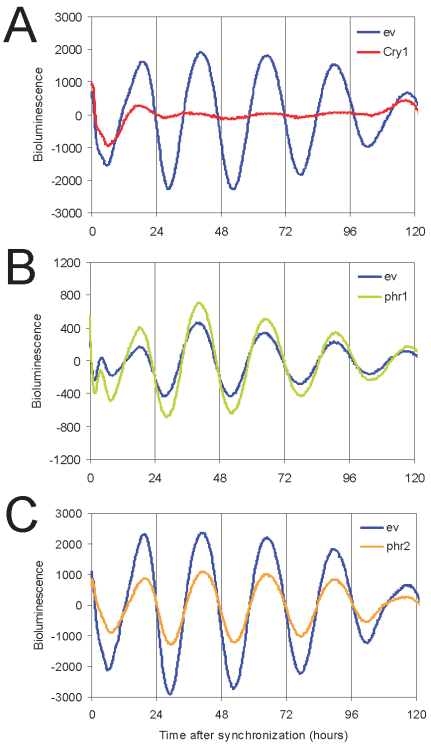

Figure 5. Circadian oscillation of synchronised ransfected NIH 3T3 cells. Representative examples of bioluminescence rhythms in synchronised NIH 3T3 cells transfected with Bmal1::luc promoter constructs. Control oscillation was monitored by co-transfection with the empty vector (ev) (blue). Co-transfection with *mCry1* is depicted in red (A), *phr1* in green (B) and *phr2* in orange (C). The values in Y-axis correspond to baseline substracted bioluminescence.

vectors, using the empty vector (ev) as control. As an additional control, we also monitored oscillations in cells transiently expressing the mCRY1 protein which, as expected, drastically reduced the amplitude of *Bmal1* rhythms (Figure 5A). The overexpression of PHR2 resulted in a significant decrease of approximately 40% (amplitude is 60±12%, p=0.02) in the amplitude of oscillations (Figure 5C), whereas overexpression of PHR1 did not significantly influence the bioluminescence rhythm (amplitude is 85±20%, p=0.2) (Figure 5B). These findings are in agreement with our observation that PHR2, but not PHR1, is able to inhibit CLOCK/BMAL1-driven transcription (Figure 3)

Discussion

In the present study, we have clearly demonstrated that the *Chrysodeixis chalcites* nucleopolyhedrovirus PHR1 and PHR2 proteins resemble mammalian cryptochrome proteins in that they can interact with CLOCK (Figure 1 and 2), a protein that is characterized as an integral component of the transcription/translation feedback loop of the mammalian circadian clock. Cryptochromes act in the molecular oscillator as repressors of theC LOCK/BMAL1 complex by interacting with CLOCK via the core domain and with BMAL1 via the predicted coiled-coil domain of CRY (Chaves et al., 2006).
As a consequence, the fact that the photolyases studied here bind actually CLOCK, but not BMAL1, is highly understandable as the predicted coiled coil domain, required for interaction with BMAL1, is absent in the baculovirus and other PHR proteins. Only PHR2, however, is capable of inhibiting CLOCK/BMAL1-driven transcription, indicating that interaction with CLOCK by itself is not sufficient to inhibit CLOCK/BMAL1-driven transcription. We have found, moreover, that overexpressed PHR2 (but not PHR1) affects the molecular oscillation in mammalian cells by decreasing the amplitude of the oscillation in cultured fibroblasts (Figure 4), which is likely due to its capacity to efficiently inhibit CLOCK/BMAL1-driven transcription (Figure 3). This finding is remarkable bearing in mind the evolutionary distance between mammals and baculoviruses.

The *phr2* gene encodes, in contrast to *phr1,* an active photolyase which can rescue photolyase-deficient *E. coli* and has CPD photolyase activity *in vitro* (van Oers et al., 2008). From sequence analyses it can be seen that PHR1 differs from other class II CPD photolyases. More importantly, these discrepancies are found in conserved regions of the sequence (van Oers et al., 2008). In the light of this observation it is not surprising that the non-functional photolyase PHR1 does not affect the molecular clock. For photoreactivation and for repression of the CLOCK/BMAL1 heterodimer, most likely, the same subdomains in the photolyase-like core domain are required. A remaining question is why ChchNPV would be equipped with two photolyase-like genes, especially if one of these, *phr1*, does not seem to have any known function. Is PHR1 only an evolutionary relict or mistake which just waits to be removed, or does it have another, yet to be discovered, function? Further studies are needed to reveal in more detail the properties of the *phr1* gene in ChchNPV and its homologs in other plusiine-infecting baculoviruses (Xu et al., 2008) to come to a better understanding of *phr1* functions.

Non-mammalian vertebrate cryptochromes (*Danio rerio*, *Xenopus leavis*, *Gallus gallus*) are able to inhibit mouse CLOCK/BMAL1-driven transcription, while none of the tested photolyases from those organisms possess this function (Kobayashi et al., 2001; Daiyasu et al., 2004; Yamamoto, Okano and Fukada 2001). Recently, a clock repression function has been reported for *Phaeodactylum tricornutum* (diatom) and

Ostreococcus tauri (green alga) (6-4)PP photolyases (Coesel et al., 2009; Heijde et al., 2010). It has been proposed that duality of function of photolyases is encountered in the lower specialized organisms, when compared to vertebrates, which is supported by our analyses of the baculovirus PHR proteins. The taxonomic position of the organism, however, is not an appropriate criterion to exclude a circadian role of photolyases as the *P. tridactylus* CPD photolyase also affects CLOCK/BMAL1 driven transcription (Chaves et al., 2010 submitted). It is accepted theory that the evolution of cryptochromes is the result of point mutations in a photolyase ancestor gene, combined with the addition of a BMAL1 interacting domain to secure clock function (Chaves et al., 2006; Zhu, Conte and Green, 2003; Balland et al., 2009). Our finding takes us another step further in revealing the evolutionary relationshipbetween members of the cryptochrome/photolyase family. Domain analyses of PHR1 and PHR2 and *in vivo* studies with mutant ChchNPVs have to be performed to pin-point differences between the viral PHRs and to obtain deeper insight into the function and evolution of baculovirus photolyases.

Although over 50 baculovirus genomes have been sequenced so far (reviewed by Harrison, 2009), only few possess a photolyase gene. From an ecological point of view, carrying an active photolyase enzyme would be highly beneficial for the insect virus. It is known that upon infection by a baculovirus, the infected larvae become hypermobile and often climb up to the top of the plant canopy, allowing efficacious spreading of the progeny virus over the foliage and highly facilitating transmission of the virus within the insect population (Goulson, 1997). As a consequence, however, the virus is more exposed to UV light, which could lead to its rapid inactivation (Sun et al., 2004). To survive such conditions some insect viruses are equipped with a photolyase with DNA repair activity towards UV-induced lesions. This is also found for the entomopox virus AMEV (Nalcacioglu et al., 2010).

Here, we have shown that the role of photolyases in virus-insect host interaction may be much broader then previously thought and may also encompass a circadian clock-related function. It is tempting, therefore, to speculate about the ecological benefit for baculoviruses to contain a photolyase with a potential clock related function. Insects were among the first organisms where a master clock was identified (reviewed by Helfrich-Förster, 1998) and there is striking similarity between the oscillators in mammals and insects. It is crucial to emphasize that both CLOCK and BMAL1 have their orthologs in insects: CLOCK and CYCLE, respectively (reviewed by Rosbach et al., 2009). We hypothesize that the circadian role of PHR2 (as identified using the mammalian clock system) is not accidental and that this protein might also affect the circadian clock of the insect host. Possibly, the photolyase encoded by the viral genome not only protects against UV light but also might have a role in virus-induced behavioural changes by influencing the host's circadian clock. This question is still open and *in vivo* studies with *phr1* and *phr2* knockout ChchNPVs in *Chrysodeixis chalcytes* larvae are needed to reveal the whole set of functions of the encoded PHR proteins.

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The authors wish to thank Dr. Kazuhiro Yagita (Osaka University Graduate School of Medicine, Japan) for kindly providing us with the *mPer2::luciferase* reporter construct and *Bmal1*-flag and *Clock-flag* expression constructs. The *Bmal1::luciferase* construct

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Supplementary Information

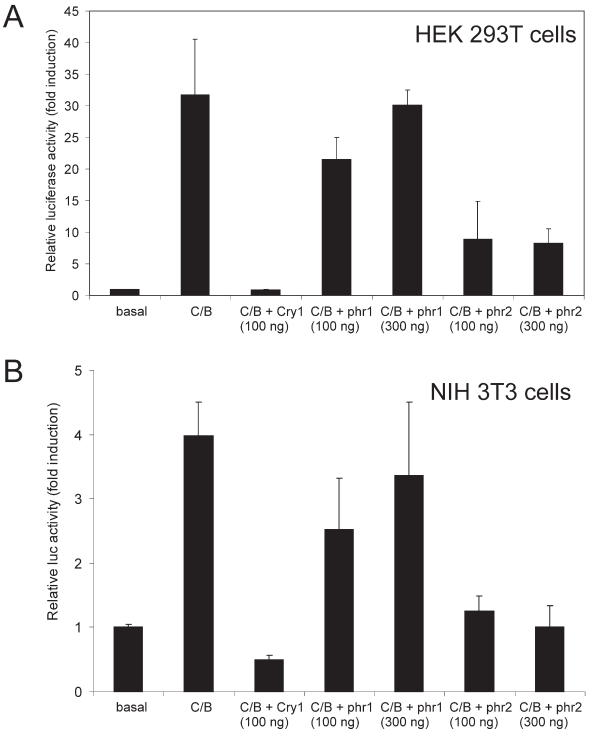

Figure S1.
Effect of PHR on transcription driven by CLOCK/BMAL1.
Relative luciferase activity in HEK 293T cells (A) and NIH 3T3 cells (B). In all reactions cells were transfected with the reporter constructs Per2:: luciferase (luminescence read-out) and pRL (as internal control). Cells were co-transfected with *Clock* and *Bmal1*, and *Clock* and *Bmal1* with either *mCry1* (100 ng), *phr1* (100 or 300 ng) or *phr2* (100 or 300 ng), as indicated under the graphic. The luciferase activity is shown as fold induction from the "basal" condition, which is set to 1. Error bars (± s.e.m.) were calculated from three independent experiments. p-values lower than 0.05 are indicated with asterisks.

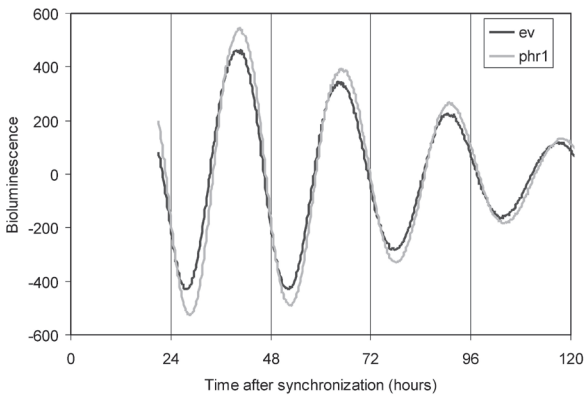

Figure S2. Effect of PHR1 on the circadian oscillation of synchronized NIH 3T3 cells. Representative examples of bioluminescence rhythms in synchronized NIH 3T3 cells (n=3). Bmal1::luc. (250 ng) was used as reporter construct to follow oscillation in real-time. The control oscillation was monitored by co-transfection with the empty vector (ev), shown in dark grey. The oscillation of cells co-transfected with *phr1* are shown in light grey. In each case 750 ng of plasmid was transfected. The values in Y-axis correspond to baseline subtracted bioluminescence values.

CHAPTER 5

The Potorous CPD photolyase rescues a cryptochrome-deficient mammalian circadian clock

Abstract
Despite the sequence and structural conservation between cryptochromes and photolyases, members of the cryptochrome/photolyase (flavo)protein family, their functions are divergent. Whereas photolyases are DNA repair enzymes that use visible light to lesion-specifically remove UV-induced DNA damage, cryptochromes act as photoreceptors and circadian clock proteins. To address the functional diversity of cryptochromes and photolyases, we investigated the effect of ectopically expressed *Arabidopsis thaliana* (6-4)PP photolyase and *Potorous tridactylus* CPD-photolyase (close and distant relatives of mammalian cryptochromes, respectively), on the performance of the mammalian cryptochromes in the mammalian circadian clock. Using photolyase transgenic mice, we show that Potorous CPD-photolyase affects the clock by shortening the period of behavioral rhythms. Furthermore, constitutively expressed CPD-photolyase is shown to reduce the amplitude of circadian oscillations in cultured cells and to inhibit CLOCK/BMAL1 driven transcription by interacting with CLOCK. Importantly, we show that Potorous CPD-photolyase can restore the molecular oscillator in the liver of (clock-deficient) *Cry1/Cry2* double knockout mice. These data demonstrate that a photolyase can act as a true cryptochrome. These findings shed new light on the importance of the core structure of mammalian cryptochromes in relation to its function in the circadian clock and contribute to our further understanding of the evolution of the cryptochrome/photolyase protein family.

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Introduction

Life is subject to the 24-hour rotation cycle of the earth, which imposes rhythmic changes in light and temperature conditions. In order to anticipate these environmental changes, most organisms have developed a circadian clock with a period of approximately 24 hours that allows them to adjust behavior, physiology and metabolism to the momentum of the day. To keep pace with the day/night cycle, this internal clock needs to be reset every day, using light (the most predictable environmental cue) as the strongest Zeitgeber (German for "time giver" or synchronizer). The mammalian master clock is located in the suprachiasmatic nuclei (SCN) of the hypothalamus, and receives light-induced signals from the retina via the retino-hypothalamic tract (Rusak et al., 1989). In turn, this master clock sends humoral and neuronal signals that synchronize peripheral oscillators, located in virtually every cell or tissue (Balsalobre et al, 1998; McNamara et al., 2001; Pando et al., 2002; Yoo et al., 2004).

The mammalian cryptochrome proteins (CRY1 and CRY2) belong to the cryptochrome/photolyase family (CPF) of flavoproteins and were initially identified as homologues of photolyase (Todo et al., 1996; van der Spek et al., 1996). In view of their strong resemblance to plant cryptochrome proteins, which act as blue light photoreceptors, the mammalian CRY proteins were hypothesized to act as photoreceptors for resetting of the circadian clock (Todo et al., 1996; Hsu et al., 1996). Unexpectedly however, inactivation of the *Cry1* and *Cry2* genes in the mouse was shown to shorten or lengthen the period length of the circadian clock respectively, whereas in the absence of both genes circadian rhythmicity was completely lost (van der Horst et al., 1999; Vitaterna et al., 1999; Okamura et al., 1999). This observation, together with the finding that the *Cry* genes encode the most potent inhibitors of the circadian transcription activator CLOCK/BMAL1 (Kume et al., 1999), positioned the mammalian CRY proteins at the heart of the circadian core oscillator.

The mammalian circadian clock consists of a molecular oscillator, composed of a set of clock genes that act in transcription-translation-based feedback loops. The CLOCK/BMAL1 heterodimer activates transcription of the *Period* (*Per1*, *Per2*) and *Cryptochrome* (*Cry1*, *Cry2*) clock genes through E-box elements in their promoter. Following synthesis, the PER and CRY proteins will gradually accumulate in the nucleus and ultimately repress CLOCK/BMAL1, and thereby transcription of their own gene (Reppert and Weaver, 2001; Young and Kay, 2001; Ko and Takahashi, 2006). A second loop is formed by REV-ERBα, which cyclically inhibits RORα-driven transcription of the *Bmal1* gene (Preitner et al., 2002; Etchegaray et al., 2003; Sato et al., 2004; Tsai et al., 2008). Adding to this transcription/translation feedback loop mechanism is a network of post-translational modifications of clock proteins (phosphorylation, (de)acetylation, sumoylation and ubiquitylation) that fine-tune the period length of the circadian oscillator and confer robustness and persistence to the molecular clock Toh et al., 2001; Yagita et al., 2002; Cardone et al., 2005; Lee et al., 2008; Asher et al., 2008; Nakahata et al., 2008; Gallego and Virshup et al., 2007; Vanselow and Kramer et al., 2007).

Photolyases, the other members of the CPF, are DNA repair enzymes that use visible light to lesion-specifically remove ultraviolet light-induced cyclobutane pyrimidine dimers (CPDs) or (6-4) pyrimidine-pyrimidone photoproducts ((6-4)PPs) from the DNA in a reaction called photoreactivation (Sancar, 2003; Eker et al., 2009). Placental mammals have lost photolyase genes during evolution and solely rely on nucleotide excision repair

for removal of CPDs and (6-4)PPs (Yasui et al., 1994). Nevertheless, when expressed in the mouse, CPD and (6-4)PP photolyases rapidly remove these UV-induced lesions in a light-dependent manner and protect the animal from sunburn, mutation induction, and skin cancer development (Schul et al., 2002; Jans et al., 2005).

Phylogenetic analysis has shown that the CPF is divided in two major subgroups. The first subgroup encompasses (i) class I CPD photolyases, (ii) (6-4)PP photolyases and animal cryptochromes, (iii) plant cryptochromes, and (iv) DASH cryptochromes, whereas the second subgroup is solely composed of class II CPD photolyases (Eker et al., 2009). It is accepted that all members of the photolyase/cryptochrome protein family evolved from a common ancestor CPD photolyase by multiple gene duplications (Kanai et al., 1997). Cryptochromes and photolyases on the one hand share a common backbone, the core domain, which binds two chromophoric cofactors (i.e. FAD and either 5,10-methenyl-tetrahydrofolate or 8-hydroxy-5-deazaflavin), but on the other hand differ in the presence of N- and C-terminal extensions (see Figure 1). Whereas eukaryotic photolyases have an N-terminal extension, containing nuclear and mitochondrial localization signals, cryptochromes contain a unique C-terminal extension of variable length and amino acid composition. It is currently accepted that the functional diversity among cryptochromes (i.e. photoreceptor, circadian photoreceptor, or core clock protein) is achieved by the diversity of their C-terminal extensions.

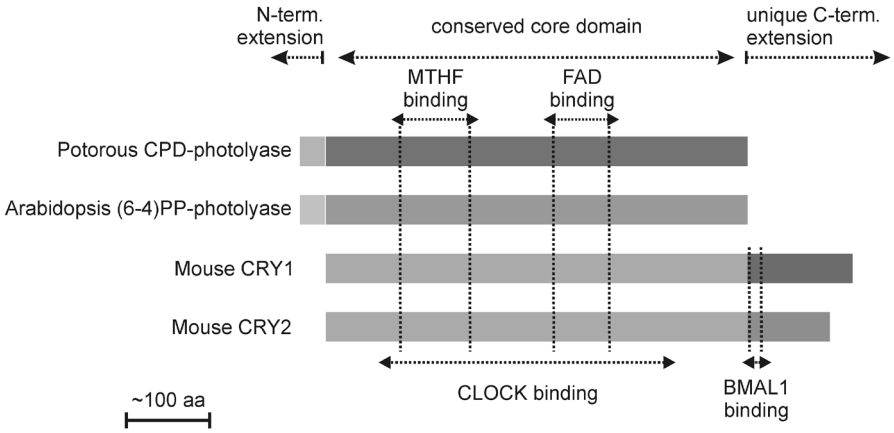


Figure 1. Cryptochromes and photolyases
Schematic representation of *Pt*CPD-PL, *At*(6-4)PP-PL and mouse CRY1 and 2. Conserved and unique domain are indicated above, chromophore binding site are indicated by vertical doted lines. CLOCK and BMAL1 binding regions in mouse CRY and indicated below.

Detailed structure/function analysis of the C-terminal region of mammalian CRY1 allowed us to identify a putative coiled-coil domain at the beginning of the C-terminal extension as a potential PER and BMAL1 binding site (Chaves et al., 2006). Deletion of the complete C-terminal extension (aa 471-606 of mouse CRY1) abolished the CLOCK/BMAL1 transcription inhibitory potential of CRY1, Similarly, Green and co-workers have demonstrated that the C-terminal extension of *Xenopus laevis* CRY proteins is crucial for transcription repression (van der Schalie et al., 2007). Interestingly, specific deletion of either the coiled-coil domain (aa 471-493) or the downstream tail region (aa 494-606) of mammalian CRY1 failed to eliminate its ability to inhibit CLOCK/BMAL1-

mediated transcription (Chaves et al., 2006), likely because these mutant proteins can still bind to CLOCK via a yet unidentified region of the core domain. This finding lead us to suggest that an interaction between the C-terminal extension and the core domain is mandatory for the clock function of mammalian cryptochromes, possibly by providing structure to the latter (Chaves et al., 2006).

In the present study, we have explored the importance of the core domain of mammalian CRY proteins for core oscillator function by addressing the question to what extent photolyase enzymes affect circadian core oscillator function. Using *in vivo* (photolyase transgenic mice) and *in vitro* (cellular clock reporter and CLOCK/BMAL1 transcription assays) approaches, we show that *Potorous tridactylus* CPD photolyase (hereafter referred to as *Pt*CPD-PL) not only displays cryptochrome-associated functions, but also can replace the CRY proteins in the mammalian circadian core oscillator.

Materials and Methods

Ethics statement

Mice were kept at the Animal Resource Center (Erasmus University Medical Center), which operates in compliance with the European guidelines (European Community 1986) and The Netherlands legislation for the protection of animals used for research, including ethical review. Animal studies at Erasmus University Medical Center were approved by DEC Consult, an independent Animal Ethical Committee (Dutch equivalent of the IACUC) under permit numbers 139-09-02 (EUR1702) 139-09-11 (EUR1760) and 139-09-12 (EUR1761).

Mouse lines and monitoring of circadian behavior

β-actin::*At(*6-4)PP-PL [33], β-actin::*Pt*CPD-PL [31], and *Per2::Luc* transgenic mice (generation described below), as well as *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ knockout mice (van der Horst et al., 1999), all in a C57BL/6J background, were housed under standard conditions and fed *ad libitum*. All mouse lines were backcrossed at least 11 times to a C57BL/6J background. For the monitoring of locomotor activity rhythms, male mice (12-16 weeks) were individually housed in a light-proof chamber in cages (30 x 45 cm) equipped with a running wheel (14 cm in diameter) and a sensor system to detect wheel revolutions. Animals were maintained in a cycle of 12 h light (150 lux) and 12 h darkness (LD) or in continuous darkness (DD) in constant ambient temperature with water and food available *ad libitum*. Voluntary wheel running (wheel revolutions per unit of time) was continuously recorded by an online computer using the ERS program. Activity records were plotted as actograms and the period of locomotor activity was determined by the chi-square method. Unpaired Student's t-tests were used to make statistical comparisons between the different genotypes.

Generation of Per2::Luc transgenic mice

The construct used to generate the *Per2::Luc* transgenic mice consists of the luciferase gene under control of the *mPer2* promoter, cloned in pBS (Supplementary Figure S1). The primers used to amplify the 4.2 kb *mPer2* promoter fragment are indicated in Supplementary Figure S1. Intronic sequences from the rabbit β-globin locus were included in the expression construct for messenger stability. The expression construct fragment was excised from the plasmid using appropriate restriction enzymes, separated

from the vector DNA by agarose gel electrophoresis, isolated from the gel with the GeneClean II kit (Bio101), and further purified using Elutip-D-mini columns (Schleicher and Schuell, Dassel, Germany). The fragment was dissolved in injection buffer (10 mM Tris–HCl pH 7.5, 0.08 mM EDTA) and injected in the pronucleus of fertilized eggs derived from FVB/N intercrosses as described (Hogan et al., 1994). Animals were backcrossed in a C57BL/6J background. Genotyping was performed by PCR using primers located in the luciferase gene (Supplementary Figure S1). Annealing was performed at 55°C. DNA derived from transgenic mice rendered a PCR product of 475 bp, whereas no product was detected using DNA from wild type litter mates.

RNA isolation and quantitative PCR

Coronal cryrosections (25 µm) mounted on 1 mm PALM pen-membraneTM slides were rapidly thawed, fixed for 30 seconds in 70% EtOH and immediately stained with haematoxylin for 3 minutes. Following staining sections were rinsed in DEPC treated dH_2O and dehydrated by several rinses in 100% EtOH. Laser catapult microdissection (LCM) of the SCN was accomplished using the PALM Microlaser system on freshly prepared sections. Isolated SCN was dissolved immediately in Lysis Buffer (Qiagen) and stored at –80°C for subsequent RNA purification. RNA was purified with the inclusion of 'on-column' DNase treatment using the Qiagen RNeasy 'Micro' kit according the manufacturers protocol, except that an additional elution was performed in the final step to maximize RNA yield. RNA eluted with RNase-free dH_2O was vacuum evaporated for immediate amplification and cDNA generation. Quality of the freshly purified RNA was assayed using the Agilent BioAnalyzer in combination with the RNA 'Pico' chip. When intact 18S/28S ribosomal RNA peaks were evident, the sample was considered worthy of assay by Q-PCR.

Amplification was accomplished using the Ovation™ RNA Amplification System V2 according to the manufactures protocols (Nugen Technologies Inc). Efficiency of the amplification was assayed quantitatively by 260/280 nm estimation of cDNA concentration, where a yield of at least 4.8 µg cDNA was deemed sufficient for specific amplification of the RNA template; and qualitatively using the BioAnalyzer with the RNA 'Nano' chip to confirm that the majority of unfragmented, amplified cDNA is approximately of 900 bp in length. Quantitative PCR for the determination of *At*(6-4) PP-PL and *Pt*CPD-PL mRNA levels was performed in triplicate using an iCycler iQ™ Real-Time PCR Detection System (BioRad), SYBR-green and primers (Schul et al., 2002; Jans et al., 2006) generating intron-spanning products of 150-300 bp. Expression levels were normalized to *Hprt* (hypoxanthine guanine phosphoribosyl transferase) mRNA levels. The generation of specific products was confirmed by melting curve analysis, and primer pairs were tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve, which was used to calculate primer pair efficiencies.

Cell culture and transfection

COS7 [35], NIH3T3 (American Type Culture Collection), and HEK293T (American Type Culture Collection) cells, as well as primary wild type and *Pt*CPD-PL mouse dermal fibroblasts (MDFs) and immortalized *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ MDFs, were cultured in Dulbecco's modified Eagle's medium-F10-Pen/Strep-10% fetal calf serum. To generate MDFs, mice were sacrificed by cervical dislocation, and a small piece of back skin of the mouse was removed and cut into pieces with a razor blade). Skin pieces were washed in ethanol,

rinsed in phosphate-buffered saline, and incubated overnight in medium supplemented with 1.6 mg/ml collagenase type II. Single cells were obtained by passing through a cell strainer, and collected by centrifugation for 5 min at 500 g, resuspended in culture medium, and seeded onto a 10 cm dish. MDFs were cultured in a low-oxygen incubator (5% CO_2, 3% O_2). Transient expression studies were performed by transfecting cells with plasmids using Fugene reagent (Boehringer) according to the manufacturer's instructions. The following pCDNA3-based plasmids (Invitrogen) were used: pcDNA-HA-mCry1, pcDNA-*Pt*CPD-PL, pcDNA-Bmal1 and pcDNA-Clock. pcDNA-*Pt*CPD-PL is based on the construct used to generate the transgenic mice (Schul et al., 2002). For luminescence measurements pGl4.11-Bmal1::luciferase (kindly provided by Dr. U. Schibler, Geneva) was used as a reporter.

Real time bioluminescence monitoring

To monitor circadian oscillations in cultured cells in real time, cells were cultured in medium buffered with 25 mM HEPES and containing 0.1 mM luciferin (Sigma). After synchronization of intracellular clocks by treatment of confluent cultures with forskolin (dissolved in 100% ethanol, added to the culture medium at a final concentration of 30 µM), bioluminescence was recorded for 7 days (75 sec measurements at 10 min intervals) with a LumiCycle 32-channel automated luminometer (Actimetrics) placed in a dry, temperature-controlled incubator at 37°C. Data was analyzed with the Actimetrics software and two sample comparisons were done using a Students T-test. Amplitudes were calculated both with Actimetrics software, on base-line subtracted data, and by comparing peak versus trough values for RAW data. Control amplitude was set at 100%. Both methods were comparable.

CLOCK/BMAL1 transcription reporter assay

To determine the capacity of *Pt*CPD-PL to inhibit CLOCK/BMAL1 driven transcription, we used a luciferase reporter assay as previously described (Kume et al., 1999; Kanai et al., 1997). COS7 cells were transfected with 200 ng of the *mPer1*::*luciferase* reporter construct and 15 ng of null-*Renilla luc*, which was used as an internal control. Clock, Bmal1, Cry1 and *Pt*CPD-PL plasmids were added as indicated in the figure legend. The total amount of DNA transfected was kept constant at 2 µg by supplementing with empty pcDNA3.1 vector (Invitrogen). Transcriptional activity was assessed with the Dual-Luciferase 10 Reporter Assay System (Promega) by measuring a ratio of firefly luciferase activity to *Renilla* luciferase activity in each cellular lysate.

Co-immunoprecipitation experiments

Co-immunoprecipitation studies were performed as described previously (Yagita et al., 2002). In short, we transiently expressed a *Pt*CPD-PL and either Flag-Bmal1 and/or Flag-Clock in HEK 293T cells and used anti-FLAG antibodies (Sigma) and anti-*Pt*CPD-PL (Schul et al., 2002) antibodies for the immunoprecipitation and immunoblot analysis step (1:1000 dilution). As secondary antibody, we used horseradish peroxidase conjugated anti-mouse IgG (DAKO) and anti-rabbit IgG (BioSource) at a 1:1000 dilution. Chemoluminescence was detected using the ECL system (Pharmacia Biotech).

Hydroporation experiments

Hydrodynamic tail vein injection experiments were performed as described (Liu et al.,

1999). In brief, a sterile Ringers Solution (0.9% NaCl, 0.3% KCl, 0.13% $CaCl_2$) containing a total of 10 µg plasmid DNA was rapidly injected (8-10 sec) in the tail vein of the mouse under isoflurane anesthesia. This induces uptake of DNA by the liver, which is initially transient and a small proportion will integrate upon regeneration of the liver. Expression of the hydroporated constructs was non-invasively analyzed using an IVIS® Spectrum imaging device (Caliper/Xenogen) (Supplementary Figure S2), and positive mice were selected for liver isolation and slicing. Animals were sacrificed 24 h after injection (transient expression) and the livers were rapidly removed and placed in ice cold Hank's balanced salt solution supplemented with 50 mM glucose, 4 mM sodium bicarbonate, 10 mM HEPES, 10.000 units/ml penicillin/10.000 µg/ml streptomycin (Yoo et al., 2004). Liver slices (200 µm) were prepared using an automated Krumdieck tissue slicer (Alabama R&D). Individual slices were placed on a membrane insert (Millipore) in a 35-mm dish in imaging medium (DMEM supplemented with 0.1 mM luciferin, 2% B27 supplement, 4 mM sodium bicarbonate, 10 mM HEPES, 2.5 ml 10.000 units/ml penicillin/10.000 µg/ml streptomycin). Real time imaging and synchronization were performed as described above. The plasmids used were pGl4.11-Bmal1::luciferase and pcCry1::PtCPD-PL. Cloning and characterization of the *Cry1* promoter will be described elsewhere (Saito and van der Horst, unpublished data).

Results

Potorous tridactylus CPD photolyase transgenic mice have a short period circadian clock

To investigate whether their strong structural resemblance to cryptochromes (see Figure 1) allows photolyases to interfere with mammalian circadian core oscillator function, we took advantage of the availability of β-actin promoter-driven *Potorous tridactylus* CPD photolyase and *Arabidopsis thaliana* (6-4)PP photolyase transgenic mice (hereafter referred to as *Pt*CPD-PL and *At*(6-4)PP-PL mice), previously generated in our laboratory. These animals carry 3 copies of the *Pt*CPD-PL and *At*(6-4)PP-PL transgene, respectively, and express an active photolyase, capable of removing DNA lesions from the DNA in a light-dependent manner (Schul et al., 2002; Jans et al., 2006). As shown in Figure 2A, and as could be expected on the basis of the ubiquitous expression of the *β-actin* promoter,

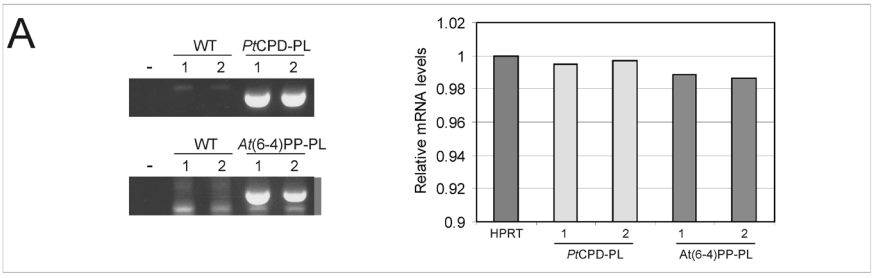


Figure 2. **Circadian behavior of photolyase transgenic mice.**
(A) Quantitative RT-PCR analysis of photolyase mRNA levels in the laser-microdissected SCN of *Pt*CPD-PL and *At*(6-4)PP-PL transgenic mice. Left panel: Ethidium bromide stained gel of PCR amplified cDNA, obtained from two independent transgenic mice and corresponding wild type littermates (sacrificed at ZT3). Right panel: graphic representation of quantitative RT-PCR amplification data from two independent animals per genotype (see Experimental procedures for details). The Y-axis represents the *Pt*CPD-PL and *At*(6-4)PP-PL mRNA levels relative to that of *Hprt*.

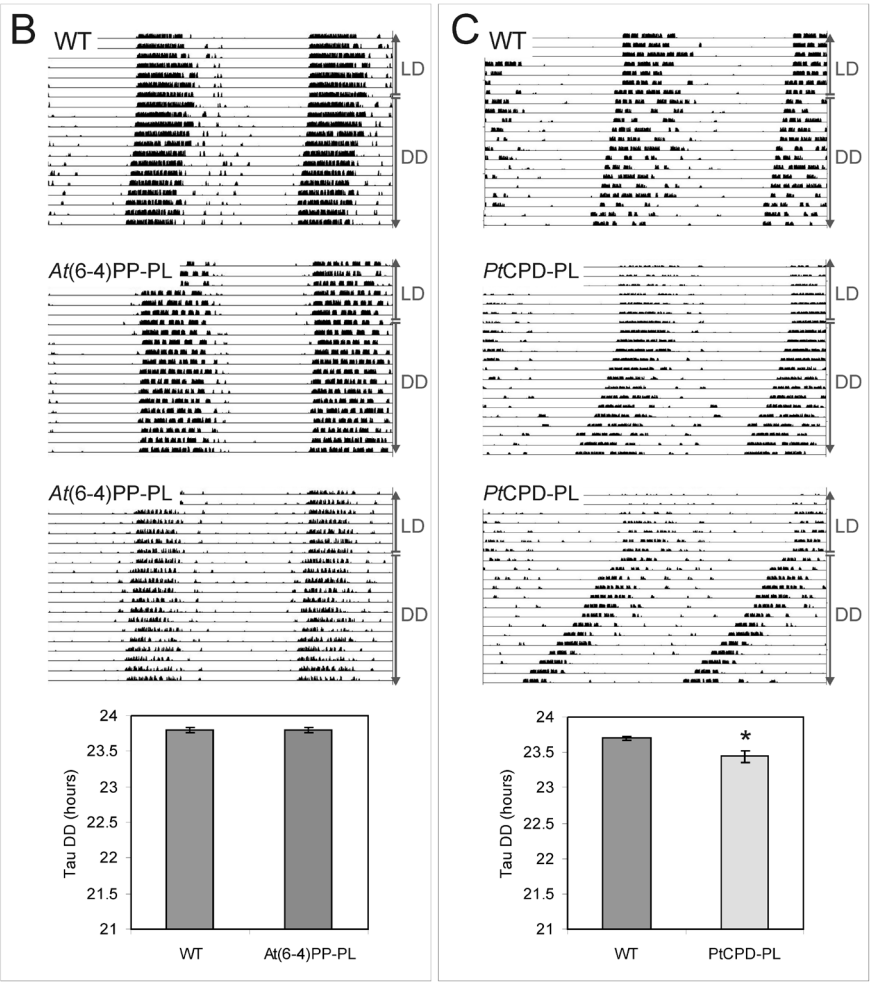


Figure 2. (B, C) Circadian behavior of *At*(6-4)PP-PL (B) and *Pt*CPD-PL (C) transgenic mice and corresponding littermates (n = 10 per genotype). Animals were kept under normal light conditions (LD 12:12 h) and subsequently exposed to constant darkness (DD) (indicated on the right side of the panels). Shown are representative examples of double-plotted actograms and graphic representations of the free-running period (τ) in constant darkness (bottom panels). Error bars represent the standard error of the mean (SEM); the asterisk indicates significance (p=0.03).

quantitative RT-PCR analysis of mRNA derived from laser microdissected SCN revealed that *Pt*CPD-PL and *At*(6-4)PP-PL mice express the photolyase transgene in the SCN at comparable levels to the *Hprt* gene. We next addressed the question whether expression of photolyase in the SCN would affect the circadian behavior of the mouse. To this end, we measured circadian wheel- running behavior of photolyase transgenic mice and sex and age-matched control littermates under normal light/dark (LD) cycles and in constant darkness (dark/dark; DD). As shown in Figure 2B, the period length (tau or τ) of circadian behavior of *At*(6-4)PP-PL transgenic mice is indistinguishable from that of the corresponding wild type littermates. In marked contrast, *Pt*CPD-PL transgenic mice

revealed a small but significant shortening of the period length of (15 to 20 min, $p<0.05$), as compared to wild type littermates (Figure 2C). In addition, the tau of the *Pt*CPD-PL mice has a larger (2-fold) variation than that of control littermates, possibly derived from small individual differences in expression levels.

These findings strongly suggest that expression of *Potorous tridactylus* CPD photolyase in the SCN interferes with circadian clock performance. In contrast, *Arabidopsis thaliana* (6-4)PP photolyase does not appear to influence the circadian clock. This observation is in agreement with our finding that *At*(6-4)PP-PL is not able to inhibit CLOCK/BMAL1 (Chaves et al., 2006). We therefore further will focus on the effect of *Pt*CPD-PL on circadian rhythms.

Potorous tridactylus CPD photolyase dampens the circadian core oscillator

As photolyases structurally resemble cryptochromes (Eker et al., 2009; Kanai et al., 1997), and given the observation that transient constitutive overexpression of the CRY1 protein suppresses the rhythmic expression of a cotransfected Bmal1::Luc reporter gene (Figure 3A), we next investigated the effect of transient overexpression of *Pt*CPD-PL on the circadian clock of cultured fibroblasts. After synchronization of the individual intracellular circadian clocks with forskolin (Balsalobre et al., 2000), cells cotransfected with the Bmal1::Luc reporter construct and empty pcDNA3 vector (used as a negative control) were shown to oscillate with a period of 25.6 ± 0.2 hr (n=11). Interestingly, overexpression of *Pt*CPD-PL reduces the amplitude of the oscillations in a dose-dependent manner (Figure 3B; Table 1). To study the influence of CPD photolyase on core oscillator performance under physiological conditions, *Pt*CPD-PL mice were interbred with *Per2::Luc* mice to obtain primary CPD-photolyase mouse dermal fibroblast (MDF) lines containing a clock reporter.

Bioluminescence rhythms in *Pt*CPD-PL/Per2::Luc MDFs show a reduction in amplitude (38 ± 5 %) when compared to those in Per2::luc (control) fibroblasts (Figure 3C; Table 1), thus confirming the data obtained in the transient expression studies. Interestingly, and in line with the animal studies, the period of oscillations in *Pt*CPD-PL/Per2::Luc MDFs (22.7 ± 0.4 hr; n=4) is approximately 50 min shorter ($p<0.05$) than that of Per2:Lluc fibroblasts that do not carry the CPD photolyase transgene (23.8 ± 0.2 hr; n=4).Taken together, these data demonstrate that the *Pt*CPD-PL exerts a dominant negative effect on the circadian clock by dampening the oscillations and shortening the period length, likely by interfering with CRY mediated functions.

Potorous tridactylus CPD photolyase inhibits CLOCK/BMAL1-driven transcription

The dominant negative effect of CPD photolyase on cellular clock performance and circadian behavior, as vident from the *in vivo* and *in vitro* studies, prompted us to investigate the underlying mode of action. Since CRY proteins are strong inhibitors of the CLOCK/BMAL1 transcription activator (Kume et al., 1999), we used a COS7 cell based reporter assay to analyze the ability of *Pt*CPD-PL to inhibit CLOCK/BMAL1-driven transcription of the *mPer1* promoter-driven luciferase reporter gene.

Consistent with previous studies (Kume et al., 1999; Chaves et al., 2006) simultaneous expression of CLOCK and BMAL1 causes a 30-fold induction in transcription of the luciferase gene, which is strongly repressed in the presence of CRY1 (Figure 4A). Interestingly, notwithstanding the fact that the protein should be expressed at high level, the *Pt*CPD-PL is also capable of significantly suppressing CLOCK/BMAL1 activity.

From these data we conclude that *Pt*CPD-PL is able to interfere with mammalian core oscillator performance by exerting a cryptochrome-like function, i.e. inhibition of CLOCK/ BMAL1-mediated transcription.

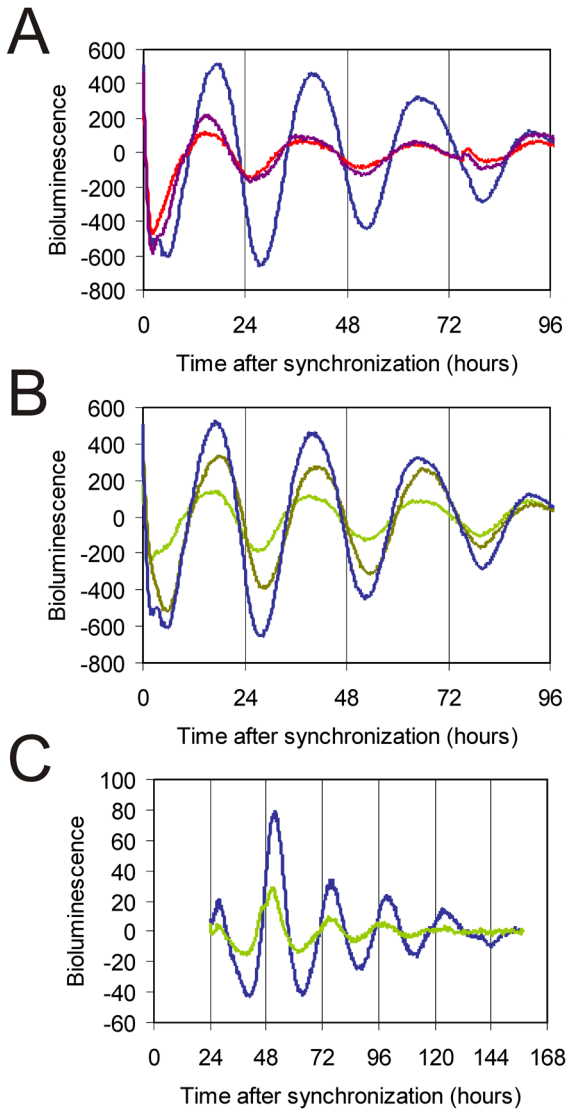


Figure 3. ***Pt*CPD photolyase dampens circadian oscillations.**

(A, B) Representative examples of bioluminescence rhythms in NIH3T3 cells co-transfected with a mBmal1::luciferase reporter construct and either empty pcDNA3 (blue line), pcDNA-Cry1, (100 ng, purple line; 200 ng, red line) or pcDNA-CPD photolyase (200 ng, olive line; 400 ng, green line). Empty pcDNA3 vector was added to correct for the amount of DNA transfected. **(C)** Representative example of bioluminescence rhythms in primary MDFs, derived from *Pt*CPD photolyase transgenic mice (green line) and wild type littermates (blue line), transiently expressing the Bmal1::luciferase reporter gene. Bioluminescence recordings were started immediately after forskolin synchronization of the individual cellular clocks. The Y-axis represents base line subtracted bioluminescence values.

***Potorous tridactylus* CPD photolyase interacts with CLOCK**

We have previously shown that the inhibition of CLOCK/BMAL1 mediated transcription by CRY1 requires a complex network of interactions with CLOCK and BMAL1, involving the CRY1 core domain and C-terminal extension (Chaves et al., 2006) (see also Figure 1). We therefore next asked the question whether *Pt*CPD-PL, like CRY proteins, can physically interact with CLOCK and BMAL1. To this end, we performed a co-immunoprecipitationexperiment using HEK293T cells overexpressing the FLAG-CLOCK or FLAG-BMAL1 proteins alone, or in combination with *Pt*CPD-PL (Figure 4B). In the

Table 1.

NIH 3T3 cells		
	Tau (hours)	*Amplitude (%)*
control	25.60 ± 0.16	100
PtCPD-PL	25.57 ± 0.16	65 ± 7

MDF lines		
	Tau (hours)	*Amplitude (%)*
WT	23.83 ± 0.18	100
PtCPD-PL	22.70 ± 0.43	62 ± 5

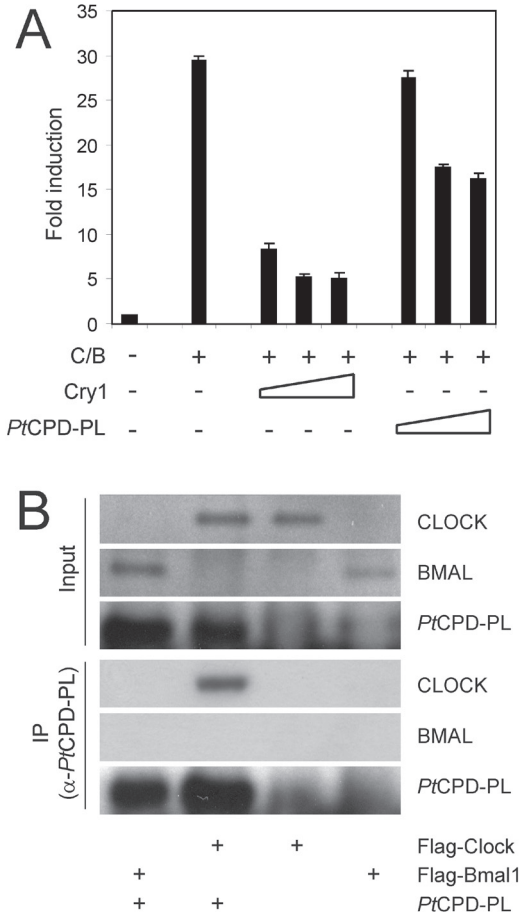


Figure 4. *Pt*CPD photolyase represses CLOCK/BMAL1-driven transcription and interacts with CLOCK. (A) COS7 cell-based CLOCK/BMAL1 transcription assay using a *mPer1* E-box promoter-luciferase reporter construct. Luminescence, shown as x-fold induction from the basal expression level (set to 1), is indicated on the Y axis. pcDNA3, pRL-CMV, and the mPer2::luc were added in all reactions. The presence or absence of Cry1 (10-100 ng) and *Pt*CPD-PL (100-300 ng) expression plasmids is indicated below the graph. Empty pcDNA3 vector was added to correct for the amount of DNA transfected. Mean and standard deviation of triplicate samples are shown. **(B)** Identification of photolyase-binding proteins. *Pt*CPD-PL was precipitated from HEK293T cells, transfected with *Pt*CPD-PL, Flag-Clock or Flag-Bmal1 or double transfected with *Pt*CPD-PL and either Flag-Clock or Flag-Bmal1. Upper panels: Immunoblot analysis of total cell lysates, confirming the presence of the various transiently expressed proteins. Lower panels (IP): Immunoblot analysis of precipitated *Pt*CPD-PL (anti-*Pt*CPD-PL antibodies) and CLOCK and BMAL1 (anti-FLAG antibodies).

absence of *Pt*CPD-PL neither CLOCK nor BMAL1 were pulled down with anti-*Pt*CPD-PL antibodies, which excludes non-specific binding of the antibodies to these proteins. However, in the presence of *Pt*CPD-PL, the CLOCK protein is shown to co-precipitate with *Pt*CPD-PL, whereas the BMAL1 protein is not. This result indicates that *Pt*CPD photolyase inhibits CLOCK/BMAL1-mediated transcription through direct interaction with CLOCK.

Potorous tridactylus CPD photolyase can replace cryptochromes in the mammalian circadian oscillator

Having shown that *Pt*CPD-PL can interact with CLOCK, leading to inhibition of CLOCK/BMAL1-driven transcription, the intriguing question arises whether this photolyase can actually replace the CRY proteins in the mammalian circadian oscillator and rescue the arrhythmicity of *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice. Generation of a new *Cry1* promoter-driven *Pt*CPD-PL transgenic mouse line in a *Cry*-deficient background and subsequent behavioral analysis would be extremely time-consuming.

As peripheral circadian clocks form a good model for the master clock in the SCN (Balsalobre et al., 1998; Yagita et al., 2001), we chose to generate stable fibroblast lines, derived from *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice, transfected with pCry1::*Pt*CPD-PL, pCry1::Cry1 or the empty vector. These cells were then transiently transfected with Bmal1::Luc reporter construct, synchronized with forskolin, and subjected to real time luminescence monitoring. As expected, expression of pCry1::Cry1 (C, D) restores circadian rhythms in arrhythmic *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ fibroblasts (Figure 5C and D) , whereas the empty vector has no effect (Figure 5A and B). Interestingly, pCry1::*Pt*CPD-PL induces oscillations (Figure 5E and F). Although the period appears in the circadian range, the irregular character of the oscillations precluded calculation of tau.Taking into account the irregular character of the above mentioned oscillations, we took an alternative approach to further to study the capacity of *Pt*CPD-PL to rescue circadian rhythms in tissues. To this end, we used the

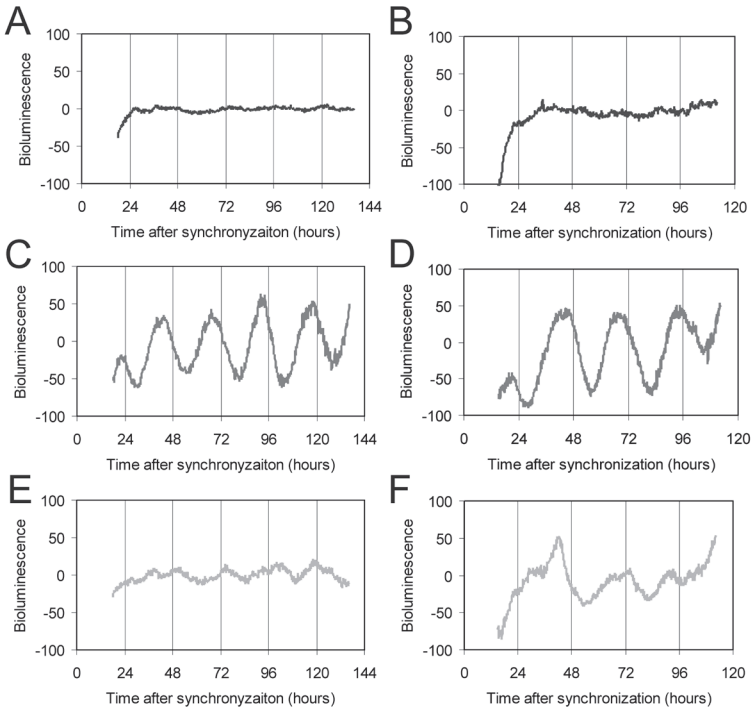


Figure 5. Correction of the circadian clock in fibroblast lines derived from CRY-deficient mice. Representative examples of bioluminescence rhythms in immortalized MDF lines, derived from *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice, and stably expressing either empty pcDNA3 **(A, B)**, pcCry1::Cry1 **(C, D)** or pcCry1::*Pt*CPD-PL **(E, F)**, and transfected with the reporter construct. Y-axis represents base line subtracted bioluminescence values.

hydroporation technique (Liu et al., 1999) to introduce clock reporter and CPD photolyase expression constructs in the mouse liver. Twenty-four hours after co-injection of pGL4.11-Bmal1::Luc and either pCry1::*Pt*CPD-PL or the empty vector in the tail vein of the mouse, liver slices were prepared and clock performance was monitored by real-time imaging of bioluminescence. Co-injection of the Bmal1::Luc reporter plasmid and empty vector in *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ mice and *Cry1*$^{+/-}$*/Cry2*$^{+/-}$ littermate controls resulted in rhythmic expression of the luciferase reporter gene in *Cry1*$^{+/-}$*/Cry2*$^{+/-}$ liver slices (Figure 6A and B), whereas, in line with the absence of a circadian clock, bioluminescence levels remained flat in liver slices from *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ mice (Figure 6C and D), thus validating the hydroporation approach. Interestingly, upon co-injection of the Bmal1::Luc reporter plasmid and pCry1::*Pt*CPD-PL and *Pt*CPD-PL expression construct in *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ animals, we observed a reinitiation of circadian rhythmicity in *Cry*-deficient liver slices for at least two cycles (Figure 6E and F). This oscillation dampened rapidly, but could be revived for at least one cycle by forskolin treatment of the liver slices (Figure 6G). As in the absence of *Pt*CPD-PL forskolin did not exert any effect on bioluminescence levels in *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ slices (Figure 6H), the forskolin-induced bioluminescence rhythm in *Pt*CPD-PL expressing *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ slices can only be explained by resynchronization of (running) intracellular clocks.

From these data we conclude that rhythmically expressed *Pt*CPD photolyase can functionally substitute for CRY proteins in the mammalian circadian oscillator and that such a *Pt*CPD-PL-driven molecular oscillator can still respond to non-photic clock-synchronizing stimuli (i.e. forskolin).

Discussion

In the present study, we analyzed the capacity of two different photolyases to interfere with circadian clock performance: the Class II CPD photolyase from *Potorous tridactylus* (*Pt*CPD-PL), which is only distantly related to CRY1, and the (6-4)PP photolyase from *Arabidopsis thaliana* (*At*(6-4)PP-PL), which is closely related to CRY1. In line with our observation that *At*(6-4)PP-PL does not inhibit CLOCK/BMAL1 transcriptional activity [35], analysis of the circadian behavior of *β-actin* promoter-driven *At*(6-4)PP-PL transgenic mice (ubiquitously expressing the transgene, including the SCN) did not reveal any dominant negative effect of the photolyase on circadian period length. In marked contrast, *β-actin* promoter-driven *Pt*CPD-PL transgenic mice showed a small but significant reduction of the period length of circadian behavior. In support of this *in vivo* observation, we also obtained a shortening of the period length of the molecular oscillator in cultured *Pt*CPD-PL dermal fibroblasts. This accelerated clock in *Pt*CPD-PL transgenic mice is unlikely an artifact resulting from unintended inactivation of known (core) clock genes, as analysis of the sequences flanking the integration site of the transgene excluded the presence of such genes (data not shown). Moreover, transiently overexpressed *Pt*CPD-PL was able to dampen the circadian oscillator in NIH3T3 cells. In this respect PtCPD-PL resembles CRY1, which when overexpressed also blunts circadian rhythms. The effect of *Pt*CPD-PL on circadian rhythms is mediated by repression of CLOCK/BMAL1-mediated transcription via direct physical interaction with CLOCK, but not with BMAL1.

Previously, we have shown that removal of the complete C-terminal extension of CRY1 abolishes repressor activity towards CLOCK/BMAL1 driven transcription of E-box containing clock genes and clock-controlled genes (Chaves et al., 2006). In the

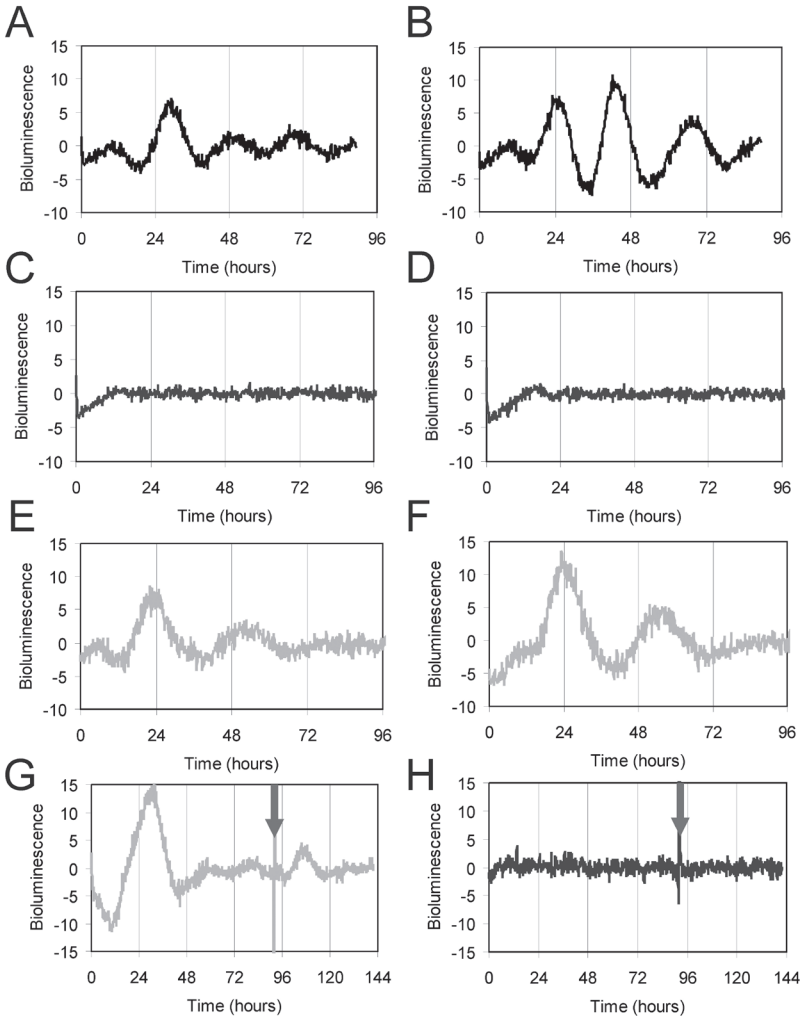


Figure 6. *Pt*CPD photolyase corrects the circadian clock in the liver of CRY-deficient mice. Representative examples of bioluminescence rhythms in liver slices obtained from mice hydroporated with pGL4.11-Bmal1::Luc (black line), together with either empty pcDNA3 (blue line) or pcDNA-CPD photolyase constructs (green line). **(A, B)** Liver slices from control mice, injected with the reporter construct only. **(C-H)** Liver slices from *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice injected with either empty pcDNA3 (C, D, H) or pcCry1::*Pt*CPD-PL (E-G), in addition to the reporter construct. In some experiments (G and H) the slices were treated with Forskolin at 96h to resynchronize the circadian clock. The Y-axis represents base line subtracted bioluminescence values.

same study, we demonstrated that whereas *At*(6-4)PP-PL by itself has no effect on CLOCK/BMAL1, fusion of the last 100 aa of the CRY1 core domain in conjunction with its C-terminal extension (aa 371-606) to *At*(6-4)PP-PL) resulted in a chimeric protein which is still able to inhibit CLOCK/BMAL1-mediated transcription. Based on these findings, we hypothesized that acquirement of C-terminal extensions (to the core domain) during evolution functionally separated cryptochromes from photolyase and conferred a clock function to the CRY proteins (Chaves et al., 2006). We now provide evidence that *Pt*CPD-PL harbors core clock features that allow it to repress CLOCK/BMAL1 transcriptional

activity and function as a true cryptochrome. Considering that *At*(6-4)PP-PL is more homologous to CRY1 than *Pt*CPD-PL, our findings suggest that it is not the primary amino acid sequence per sé, but rather the overall structure of the core domain, that makes a photolyase repressing CLOCK/BMAL1-mediated transcription. Our results indicate that the *Pt*CPD-PL by itself has the proper structure, whereas *At*(6-4)PP-PL gains such a structure after fusion with a C-terminal extension of mammalian CRY1 (Chaves et al., 2006). In addition, as *Pt*CPD-PL fails to bind BMAL1, interaction with CLOCK is sufficient to inhibit CLOCK-BMAL1-mediated transcription, which is in complete agreement with our previous observation that the BMAL1-binding coiled-coil domain in the C-terminal extension of CRY1 can be deleted without major consequences (Chaves et al., 2006).

Given the dominant negative effect of constitutive *Pt*CPD-PL expression on circadian behavior of the mouse (*in vivo* data), it is tempting to speculate on the underlying molecular mechanism by which *Pt*CPD-PL can inhibit CLOCK/BMAL1-driven transcription (*in vitro* data) and its impact on circadian core oscillator performance. By interacting with CLOCK, *Pt*CPD-PL may prevent the formation of CLOCK/BMAL1 heterodimers and/or binding of the CLOCK/BMAL1 heterodimer to E-box promoters in the DNA. In this scenario, the photolyase reduces the efficiency at which E-box containing clock (controlled) genes are transcribed by reducing the number of available transcription activators. However, as we have shown that a *Chrysodeixis chalcites* nucleopolyhedroviral photolyase can bind to mammalian CLOCK without affecting CLOCK/BMAL1 transcription potential, binding of a CPF protein per se does not prevent CLOCK/BMAL1 heterodimerization and DNA binding (Biernat and Chaves, submitted for publication). Therefore, a more plausible explanation would be that binding of *Pt*CPD-PL to CLOCK inhibits transcription activation of E-box promoter-bound CLOCK/BMAL1 heterodimers in a cryptochrome like manner. Strikingly, using an *in vivo* hydroporation approach, we show that *Pt*CPD-PL can rescue the lost circadian oscillator in CRY-deficient cells. When expressed from the *Cry1* promoter, *Pt*CPD-PL revived rhythmic expression of the *Bmal1::luciferase* reporter gene in the *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ mouse liver explants. Moreover, the period of oscillations was in the same range as that of a CRY-driven oscillator and responds to non-photic phase synchronizing stimuli (i.e. forskolin). We therefore conclude that the *Pt*CPD-PL protein has the potential to act as a true mammalian CRY protein.

While this work was in progress, two other members of the CPF have been shown to maintain dual functions: the *Pt*CPF1 protein from the marine diatom *Phaeodactylum tricornutum* and the *Ot*CPF1 protein from the green algae *Ostreococcus tauri.* These proteins hold (6-4)PP photolyase activity and (like *Potorous* CPD-PL) can inhibit CLOCK/BMAL1 driven transcription in a heterologous mammalian system (Coesel et al., 2009; Heijde et al., 2010) and are therefore considered a missing link in evolution. Interestingly, we found that *Arabidopsis thaliana* (6-4)PP photolyase does not inhibit CLOCK/BMAL1 (Chaves et al., 2006). or affect circadian behavior, whereas the distantly related *Potorous* CPD photolyase does. Moreover, we show that this marsupial class II CPD-photolyase can actually substitute for CRY proteins in the mammalian circadian oscillator. In a parallel study, we have shown that the *Chrysodeixis chalcites* nucleopolyhedrovirus PHR2 protein, another class II CPD photolyase, is able to interact with CLOCK and affect circadian rhythms *in vitro* (Biernat and Chaves, unpublished data).

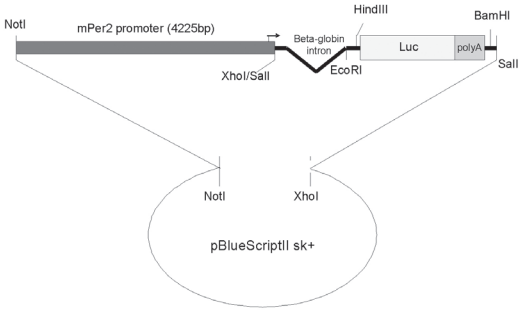
These findings contribute to understanding the functional evolution of cryptochromes and photolyases. So far, the identified CPF members with a dual function are either (6-4)PP photolyases from lower eukaryotes (Coesel et al., 2009; Heijde et

al., 2010) or class II CPD photolyases (this manuscript; Biernat and Chaves, submitted for publication). Ancestral CPF members were likely proteins with both DNA repair and circadian clock function. We propose that after the divergence of classes I and II, class II CPD photolyases have kept this dual function throughout evolution. Class I CPD photolyases and (6-4)PP photolyases, however, have lost the circadian function in time, which was taken by cryptochromes. In view of this hypothesis, it will be challenging to study molecular clocks in organisms that have both a photolyase with a dual function and cryptochromes, as is the case of marsupials, such as *Potorous* and *Monodelphis*. The genome of *Monodelphis domesticus* has been sequenced and reveals the presence of cryptochrome genes, as well as photolyase. It will be of interest to determine how the clock of non-placental mammals will respond to the loss of photolyase. On the basis of our data, one would predict a change in tau, suggesting that the circadian clock of placental mammals has adapted to the loss of photolyase by adjusting period. Studying the marsupial circadian system at the cellular and molecular level will answer these questions and shed light on the functional evolution of the CPF.

The present study has identified the *Potorous tridactylus* CPD photolyase as an attractive candidate for further structure-function studies, aiming at understanding the functional diversity between cryptochromes and photolyases. Analogous to our previous study with mammalian CRY1 - *Arabidopsis* (6-4)PP photolyase chimeric proteins, it will be informative to swap domains between *Potorous* CPD photolyase and CRY proteins for functional mapping. Such studies will ultimately reveal how nature uses the same core sequence for completely different functions (e.g photoreactivation by photolyases vs clock function of cryptochromes).

Supplementary Information

A mPer2::Luc Tg vector



B mPer2 promoter primers

5` primer: 5`- AGCGGCCGC ATTTGGGAATGTCTTGTGCGAAG -3`
NotI

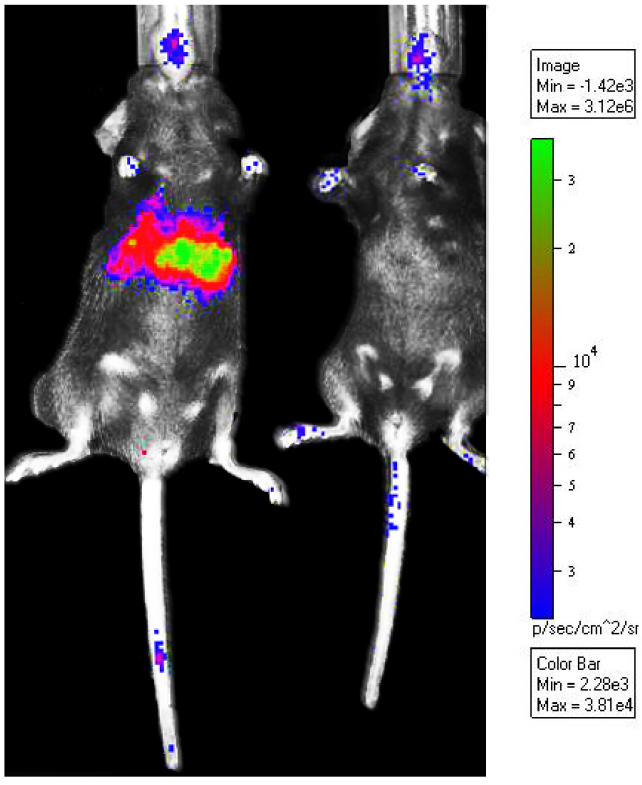
3` primer: 5`- ACTCGAG CCGCTAGTCCCAGTAGCG -3`
XhoI

C Luciferase genotyping primers

5` primer: 5`- CGAGTCGTCTTAATGTTAAGATT-3`

3` primer: 5`- TGTAGCCATCCATCCTTGTC-3`

Supplementary Figure S1. Schematic representation of the mPer2::Luc construct used to generate transgenic clock reporter mice. (A) The luciferase gene is cloned in front of the *mPer2 promoter,* using pBS as backbone. Intronic sequences from the rabbit *ß-globin* locus were included in the expression construct for messenger stability. Restriction enzyme sites are indicated. **(B)** Sequence of the primers used to amplify the 4.2 kb *mPer2* promoter fragment. **(C)** Sequence of the luciferase primers used to genotype mPer2::Luc mice.



Supplementary Figure S2. Detection of luminescence in the liver of hydroporated mice. Representative examples of dorsal luminescence images, obtained 24 hour after hydroporation of mice with either the Bmal1::Luc reporter construct (left) or the empty vector (right). Expression of the hydroporated constructs was non-invasively monitored in isoflurane anesthetized animals using an IVIS® Spectrum imaging device (Caliper/Xenogen). Colors indicate signal intensity. Note that the reporter is prominently expressed in the liver.

CHAPTER 6

Class II CPD photolyases can replace mammalian cryptochromes and can act via a similar mechanism

Abstract
Despite sequence and structural conservation, members of the cryptochrome/photolyase family (CPF) exhibit various functions. Photolyases are DNA repair enzymes, whereas cryptochromes can act as photoreceptors or transcriptional repressors in the circadian clock. The functional diversity between photolyases and cryptochromes is being questioned as some photolyases can act in the circadian clock in a manner similar to cryptochromes. It is still unclear why some photolyases possess a (partial) circadian repressor function and what molecular mechanism underlies the action of clock-functional photolyases. Here we address these questions and show that Cc-PHR2, a class II CPD photolyase from the baculovirus *Chrysodeixis chalcites* nucleopolyhedrovirus, can substitute for CRY proteins in a CRY-deficient (clock deficient) mammalian circadian oscillator. Cc-PHR2 can revive the oscillatory capacities in the liver of arrthythmic *Cry1-/-/Cry2-/-* double-knock-out mice, as well as in *Cry1-/-/Cry2-/-* mouse dermal fibroblasts. Our results shed light on the molecular mechanism underlying the action of Cc-PHR2 and PtCPD-PL, another class II CPD-photolyase (derived from the marsupial *Potorous tridactylus*).

A manuscript is in preparation by Biernat M. A., Nijman R.M., Eker A. P.M., Carvalho da Silva A.M., van Oers M. M., Vlak J. M., van der Horst G. T. J. and Chaves I.

Introduction

Light affects organisms in various ways and light-signalling is mediated by the action of different proteins, among which the members of the cryptochrome/photolyase family (CPF) of flavoproteins. The CPF consists of proteins that comprise different functions and is phylogenetically divided into two groups. Group (i) comprises class I CPD photolyases, (6-4)PP photolyases, Cry-DASH and both animal and plant cryptochromes, and group (ii) harbors class II CPD photolyases (Kanai et al., 1997). CPD photolyases and 6,4-PP photolyases catalyse a process that lesion-specifically removes UV light-induced DNA damage: cis-syn-cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6,4)-pyrimidone photoproducts (6-4)PP, respectively (Müller and Carell, 2009). Photolyases can be found among almost all organisms, including certain animal and insect viruses (Afonso et al., 1999, 2000; Willer et al., 1999; van Oers et al., 2004; Nalcacioglu et al., 2010), but are not present in placental mammals. Cryptochromes are responsible for regulating circadian clock in animals and plants (Kume et al. 1999; van der Horst et al., 1999), growth and development in plants (Cashmore et al., 2003), and are considered to be involved in magnetoreception in birds and insects (Gegear et al., 2010). It is accepted that all members of the photolyase/cryptochrome protein family evolved from a common ancestor CPD photolyase by multiple gene duplications (Kanai et al., 1997). The most likely evolutionary scenario postulates that animal cryptochromes are derived from (6-4)PP photolyases, whereas plant cryptochromes are derived from CPD photolyases (reviewed by Cashmore et al., 1999).

The CPF members are proteins that possess a conserved core domain to which two cofactors are non-covalently bound: the catalytic co-factor flavin adenonucleotide (FAD) and the antenna pigment 5,10-methylentetrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyl-5-deazariboflavin (8-HDF) (Sancar et al., 2003). Additionally, photolyases possess an N-terminal extension that contains nuclear and mitochondrial localization signals, whereas cryptochromes (except Cry-DASH) are equipped with a C-terminal extension that may differ in its length and composition (Daiyasu et al., 2004). This C-terminal domain is involved in signal-transmission and protein-protein interactions, and the sequence variability in the C-terminus is the most likely reason for diversity in cryptochrome functions (Chaves et al., 2006).

The mammalian cryptochromes CRY1 and CRY2 are part of the circadian clock that regulates the behaviour, physiology and metabolism and keeps pace with 24 h earth rotation cycle (van der Horst et al., 1999). Like most circadian clocks, both the mammalian and insect clocks are composed of a molecular oscillator with an endogenous periodicity of approximately 24 h, that acts through transcription/translation-based positive and negative feedback loops (TTFL) in which cyclic expression of clock genes is regulated by their own gene products (reviewed by Dunlop et al., 2007). In the mammalian circadian clock, the positive loop consists of a CLOCK and BMAL1 heterodimeric transcription factor, which via binding to the E-box elements in the promoter region of the genes for Cryptochromes (*Cry1* and *Cry2*) and Period (*Per1* and *Per2*) mediates transcription of these genes and other Clock-Controlled Genes (CCGs). After CRY and PER are produced, they accumulate in the cytoplasm where they form complexes that are translocated to the nucleus. In the negative loop, the CRY/PER complex inhibits CLOCK/BMAL1-driven transcription, and hence, transcription of their own genes (Ko and Takahashi, 2006). In order to assure proper clockwork there are parallel feedback loops like the Rev-erbα/β

that negatively feeds back on BMAL1 transcription (Preitner et al., 2002).

Recently, studies on (6-4)PP photolyases (Coesel et al., 2009; Heijde et al., 2010) and two class II CPD photolyases (i.e. *Pt*CPD-PL from the marsupial *Potorous tridactylus* and the Cc-PHR2 from the baculovirus *Chrysodeixis chalcites* nucleoplyhedrovirus (ChchNPV) (van Oers et al., 2008)) addressed the functional diversity between cryptochromes and photolyases (Chaves et al., 2006; Chaves et al., 2011; Biernat et al., 2012). It was shown that photolyases can partially mimic the function of cryptochrome in the regulation of the mammalian circadian clock. There is, however, limited comprehension of how and why the functional diversity among CPF members developed in the course of evolution and how some photolyases can work in both the circadian clock and DNA repair.

In this study, we further explored the clock regulatory function of the CPD photolyase from ChchNPV (Cc-PHR2) and investigated whether Cc-PHR2, similarly to *Pt*CPD-PL (Chaves et al., 2011), is able to correct a CRY-deficient oscillator. We further investigated the possible molecular mechanisms that could be employed by Cc-PHR2 and *Pt*CPD-PL in the transcription/translation feedback loop to act in the mammalian circadian clock.

Materials and methods

Plasmids and construction of plasmids

To drive cyclic transcription of photolyases, the human cytomegalovirus (CMV) promoter of pcDNA3.1 (Invitrogen) was replaced by the mouse *mCry1* promoter region, resulting in expression vector pCry1. The cloning and characterization of the *mCry1* promoter will be described elsewhere (Saito and van der Horst, unpublished data). The Cc-PHR2 coding sequence was subcloned from pcDNA3.1-PHR2 (Biernat et al., 2012) using NheI and BamHI restriction sites generating pCry1::PHR2. Generation and characterisation of pCry1::HA-CRY1, carrying the HA- mCRY1 under *mCry1* promoter region, has been described previously (Chaves et al., 2011). Additionaly, the following plasmids were used for immunoprecipitation and/or hydroporation experiments: pcDNA-HA-mCry1 (described in Chaves et al., 2011), pcDNA3.1-PHR2, pcDNA-Bmal1-FLAG and pcDNA-Clock-FLAG, all having as backbone pcDNA3 (Invitrogen). For real time bioluminometry, we used the pGl4.11-Bmal1::luciferase circadian clock reporter construct. All these plasmids were described previously (Biernat et al., 2012). Chromatin-immunoprecipitation (ChIP) and co-immunoprecipitation (Co-IP) experiments were performed using the *Pt*CPD-PL, class II CPD photolyase from *Potorous tridactylus,* in pcDNA3 and pcDNA3.1-PHR2.

Cells, culture and transient transfection conditions

HEK293T (Human Embryonic Kidney) (ATCC) and *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ mouse dermal fibroblasts (MDFs) (Chaves et al. 2011) were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)/Ham's-F10 medium (1:1; Lonza), supplemented with 10% fetal calf serum (Lonza), penicillin and streptomycin. Stable expression of mCRY1 and Cc-PHR2 was achieved by transfecting *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ MDFs with aforementioned constructs using FuGene reagent according to the manufacturer's instruction (Roche), followed by hygromycin B selection (Roche, 100 µg/ml) for two weeks. Hygromycin resistent colonies were isolated and subsequently cultured under hygromycin B selection conditions (50 µg/ml).

Real time bioluminescence

For real-time bioluminescence monitoring of circadian oscillation, *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ MDFs stably expressing either pcDNA (used as a control), mCRY1, or Cc-PHR2 were transfected with the pGl4.11-Bmal1::luciferase reporter construct. Two days after transfection, the cellular clocks were synchronized by replacing the medium with 2 ml fresh fresh medium containing forskolin (30 μM), luciferin (100 μM), HEPES (25 nM), 10% fetal calf serum and penicillin (100 units/ml) and streptomycin (100 μg/ml). After sealing with a glass coverslip and parafilm, dishes were placed in a LumiCycle 32-channel automated luminometer (Actimetrics) in a dry, temperature-controlled incubator at 37°C. Real time bioluminescence recording (60 sec measurements at 10 min intervals) and data processing have been described elsewhere (Oklejewicz et al, 2008).

Chromatin-imunoprecipitation (ChIP)

Chromatin-immunoprecipitation (ChIP) studies were performed using the Chromatin Immunopreciptitaion Assay Kit (Millipore) according to the manufacturer's protocol. Briefly, approximately 1×10^6 HEK293T cells were transfected with *Clock-flag* in combination with either *Cc-phr2, Pt*CPD-*Pl, Ha-Cry1* or empty vector. After 24-48 h, cells were treated with 1% paraformaldehyde (10-15 min, 37°C) to crosslink DNA/proteins, after which cells were washed twice with ice-cold PBS containing protease inhibitor cocktail (Roche). After harvesting, cells were lysed in SDS lysis buffer (Chromatin Immunoprecipitation Assay Kit, Milipore) and DNA was sheared by sonication (Soniprep 150, Beun De Ronde B.V.) for 5×10 s at amplitude 6 with breaks of 45 s. Lysates were cleared by centrifugation, diluted in ChIP dilution buffer and precleared with salmon sperm DNA/protein A-agarose (Chromatin Immunoprecipitation Assay Kit, Milipore), after which "Input DNA" was collected. Protein-DNA complexes were immunoprecipitated with anti-CLOCK antibodies (Abcam) overnight at 4°C. Subsequently, antibody-protein-DNA complexes were captured using salmon sperm DNA/protein A-agarose beads for 1 h at 4°C. After washing the beads with low and high salt, LiCl, and TE buffers (Chromatin Immunoprecipitation Assay Kit, Milipore), the protein/DNA complexes were eluted by boiling in 2× Laemlii buffer for 10 min. The samples were subjected to 4-12% gradient SDS-PAGE (Invitrogen). Western Blot analyses were carried out using anti-FLAG (Sigma, 1:1000 dilution), anti-PHR2 (van Oers et al., 2008, 1:500 dilution), anti-*Pt*CPD-PL (Chaves et al., 2011, 1:500 dilution) and anti-HA antibodies (Roche, 1:1000 dilution). As secondary antibodies, we used horseradish peroxidase conjugated anti-mouse IgG (DAKO), anti-rabbit IgG (Biossource) and anti-rat IgG at a 1:5000 dilution, visualized using the ECL system (Pharmacia Biotech).

Co-immunoprecipititon (Co-IP)

Co-immunopercipitation was performed as described previously (Biernat et al., 2012). Briefly, HEK293T cells were transfected with *Clock-flag* and *Bmal1-flag* in combination with either *Cc-phr2, Pt*CPD-*Pl, Ha-Cry1* or empty vector. After 24-48 h, cells were collected in lysis buffer composed of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1mM EDTA, and supplemented with a complete protease inhibitor cocktail (Roche). After centrifugation, a small amount of material was stored (input) and, following a preclearing step with Protein A-beads (GE healthcare) for 3 h at 4 ^{o}C, the remainder of the lysate was used for immunoprecipitation. The samples incubated overnight with anti-CLOCK antibody (Abcam) and subsequent incubation for 1 h at 4 ^{o}C with Protein

A-beads in order to capture antibody-protein complexes. Next, the beads were washed twice with IP wash buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0, 1% NP-40 and 0,05 % sodium deoxycholate). The input and the beads samples were boiled in 2 x Laemmli buffer and separated by SDS polyacrylamide gel electrophoresis in 4-12% gradient SDS-PAGE gels (Invitrogen). Immunoblot analysis and chemoluminescence detection were performed as described above.

Hydroporation experiments

Hydrodynamic tail vein injection experiments were performed as described previously (Chaves et al., 2011). The plasmids used were pGl4.11-Bmal1::luciferase, pcCRY1::PHR2 and pcCRY1::HA-Cry1. Real time imaging and synchronization were performed as described above.

Ethics statement

Mice were kept at the Animal Resource Center of the Erasmus University Medical Center, which operates in compliance with the European guidelines (European Community 1986) and The Netherlands legislation for the protection of animals used for research, including ethical review. Animal studies at Erasmus University Medical Center were approved by DEC Consult, an independent Animal Ethical Committee (Dutch equivalent of the IACUC) under permit number 139-09-02 (EUR1702).

Results

Cc-PHR2 restores a Cry-deficient arrhythmic circadian oscillator

We have previously shown that Cc-PHR2 and *Pt*CPD-PL can physically interact with CLOCK and inhibit CLOCK/BMAL1-mediated transcription of E-box genes (Biernat et al., 2012; Chaves et al., 2011). Moreover, PtCPD-PL can substitute for mammalian cryptochromes in the mammalian oscillator, as evident from its ability to restore the clock in the arrhythmic *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ double knock-out cells and tissues (Chaves et al., 2011). We therefore tested whether Cc-PHR2 has similar properties and could rescue the arrhythmicity of mouse dermal fibroblasts (MDFs) derived from such mice. Stable *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ fibroblast lines expressing *phr2*, *Cry1* or empty vector (ev) from mCry1 promoter after transient transfection with a Bmal1::Luc reporter construct were synchronized with forskolin and subjected to real time luminescence monitoring. Transfection of the empty vector did not affect the arrhythmic of *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ fibroblasts, while the pCry1::Cry1 plasmid, as expected, restored circadian rhythms with a period of 24.1h (Figure 1). The pCry1::PHR2 plasmid also restored, the oscillations of arrhythmic *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ fibroblasts (Figure1), but with a period in the range of 48h.

As the period of oscillations was longer than 24 h in fibroblast cell lines, an alternative approach was used to examine Cc-PHR2 for its capacity to rescue circadian rhythms. To this end, we expressed Cc-PHR2 in the liver of *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ mice using the hydroporation technique (Chaves et al., 2011). The pGL4.11-Bmal1::Luc and either pCry1::PHR2 or empty vector were injected in the tail vein of the a *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ mouse and 24 hour later the liver of this mouse was isolated, slices were cultured and expression of the reporter construct was followed by real-time bioluminescence measurements. Co-injection of the Bmal1::Luc reporter plasmid and empty vector in a double knockout mouse resulted in flat bioluminescence levels (Figure 2), meaning that the injection

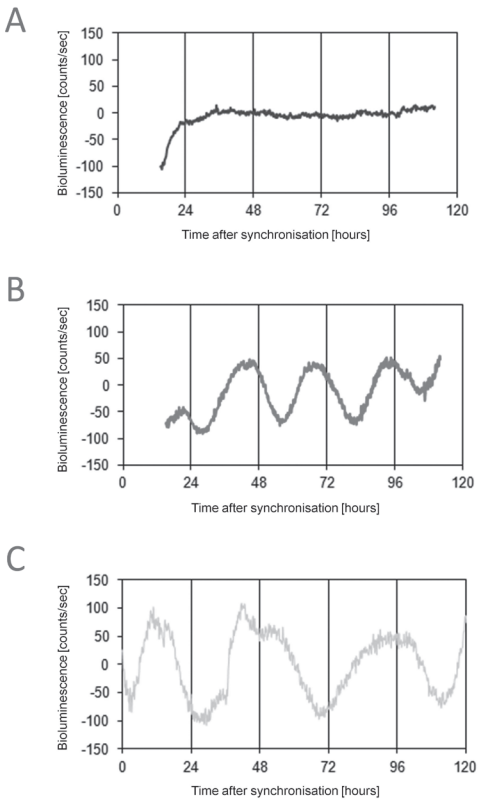


Figure 1. Cc-PHR2 restores a Cry-deficient arrhythmic circadian oscillator. Bioluminescence rhythms of forskolin synchronized mouse dermal fibroblasts (MDFs) derived from *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mouse after transient transfection with pGL4.11-Bmal1::Luc and stably expressing pCry1 empty vector (A), pCry1::HA-CRY1 (B) and pCry1:: PHR2 (C).

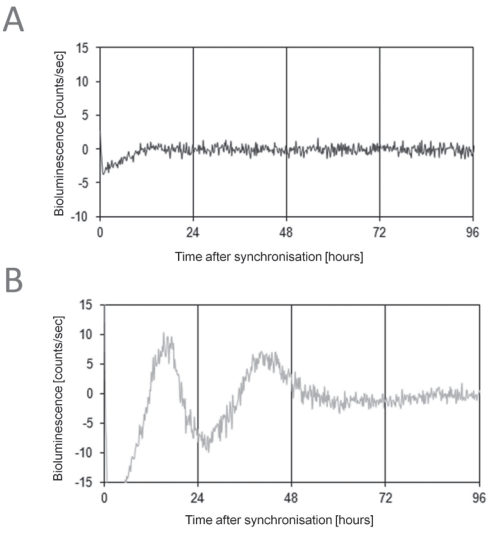


Figure 2. Cc-PHR2 restores a Cry-deficient arrhythmic circadian oscillator. Bioluminescence rhythms of forskolin synchronized liver slices derived from *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice after hydroporation with pGL4.11-Bmal1::Luc together with either empty pCry1 vector (A), or pCry1::PHR2 (B).

itself does not induce circadian rhythms. When injecting the Bmal1::Luc reporter and pCry1::PHR2 plasmids circadian rhythmicity was observed for two cycles (Figure 2) with a tau close to 24 h.

We therefore conclude that, when rhythmically expressed from mCry1 promoter, *Cc-phr2* can replace CRY proteins in the mammalian circadian oscillator.

Cc-PHR2 and Pt*CPD-*PL do not prevent binding of CLOCK to E-box-containing promoters

It was shown that Cc-PHR2 and *Pt*CPD-PL can bind CLOCK but not BMAL1, and thus, can act in the mammalian circadian clock resembling mammalian cryptochromes (Biernat et al., 2012; Chaves et al., 2011). A chromatin-immunoprecipitation (ChIP) assay was performed to identify the mechanism by which CPD photolyases could functionally resemble cryptochromes and rescue the *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ phenotype. In this approach we check whether photolyases could use the mechanism that is employed by PER and TIM to inhibit CLOCK and CYCLE to regulate the TTFL in the *Drosophila* circadian oscillator (Yu et al., 2006). We therefore studied whether photolyases, by interaction with CLOCK, could prevent CLOCK from binding to the DNA. To that aim Cc-PHR2, *Pt*CPD-PL or HA-CRY1 were co-expressed with CLOCK-FLAG. The DNA-CLOCK-FLAG complexes were immunoprecipitated with anti-CLOCK antibodies. In the presence of CRY1 we could detect, as expected, the CLOCK protein in complex with DNA (Figure 3, A). When co-expressing CLOCK with CPD photolyases, the same was observed as for CRY1 (Figure 3, A). The CLOCK protein was also detected at comparable levels, which suggests that CPD photolyases do not work in the mammalian circadian oscillator by preventing CLOCK from binding E-box elements in the chromatin.

Cc-PHR2 and Pt*CPD-*PL do not prevent formation of the CLOCK/BMAL1 heterodimer

In order to further study how the Cc-PHR2 and *Pt*CPD-PL work to rescue the *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ arrhythmic phenotype and inhibit the CLOCK/BMAL1-mediated transcription (Chaves et al., 2011; Biernat et al., 2012), a co-immunoprecipitation (Co-IP) study was performed using HEK293T cells overexpressing CLOCK-FLAG and BMAL1-FLAG alone or in combination with PHR2, *Pt*CPD-PL or CRY1-HA (Figure 3, B). CLOCK and BMAL1 were pulled down as a complex using CLOCK antibodies, both when expressed alone or in the presence of CRY1. In the presence of PHR2 or *Pt*CPD-PL, BMAL1 was as well pulled down (Figure 3, B). This result indicates that the tested class II CPD photolyases do not interfere with the binding of CLOCK to BMAL1. The interaction of CLOCK and CPD photolyases does not result, therefore, in the disruption of CLOCK/BMAL1 complex.

Discussion

In this study we analyzed the ability of Cc-PHR2 to substitute for CRY proteins in the mammalian circadian clock and examined the underlying molecular mechanisms allowing Cc-PHR2 and *Pt*CPD-PL to act in the mammalian circadian clock. Using either *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ MDFs stably expressing *mCry1* or *Cc-phr2* from the *Cry1* promoter or liver slices from *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ mice, transiently expressing these proteins, we showed that Cc-PHR2 is able to restore the oscillating expression of a Bmal1::luciferase reporter gene in a CRY-less, arrhythmic system. In fibroblasts, the period of the oscillations was, however, longer (up to around 48 h) than the circadian 24 h range whereas in mice the observed oscillation was within the circadian 24 hours range. It is possible that such a long tau, on

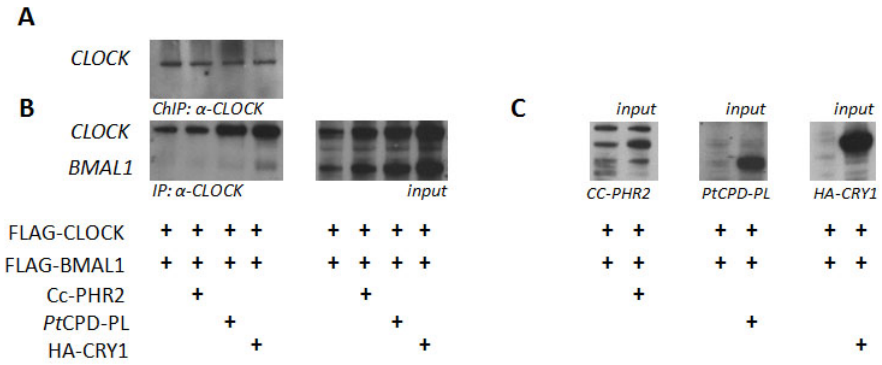


Figure 3. Class II CPD photolyases do not prevent binding of CLOCK to chromatin nor formation of the CLOCK/BMAL1 heterodimer. A. Chromatin immunoprecipitation. Immunoblot analysis of CLOCK-DNA precipitated complex. FLAG-CLOCK in complex with DNA was precipitated with anti-CLOCK antibodies from HEK293T cells transiently transfected with *Clock-Flag* and *Bmal-Flag* and either *phr2*, *PtCPD-Pl* or *Ha-Cry1*. B. Co-Immunoprecipitation. FLAG-CLOCK was precipitated with anti-CLOCK antibodies from HEK293T cells transiently transfected with *Clock-Flag* and *Bmal1-Flag* and either *phr2*, *PtCPD-Pl* or *Ha-Cry1*. The presence of BMAL1-FLAG was identified by the usage of anti-FLAG antibodies. C. The presence of expressed proteins, from left: Cc-PHR2, PtCPD-PL and HA-CRY1.

the one hand, results from higher stability of Cc-PHR2 protein in the cultured fibroblasts compared to mice. On the other hand, the long period elicited by Cc-PHR2 in the Cry-less oscillator, could indicate that PHR2 works via a completely different mechanism than the one implemented by cryptochromes to inhibit CLOCK/BMAL1-driven transcription. It is even more intriguing, when compared with *Pt*CPD-PL behaviour in the same systems (Chaves et al., 2011). *Pt*CPD-PL in *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ MDFs has a weak irregular oscillation, but within the 24 h-range. Taking all results into account, it is tempting to speculate that both CPD photolyases can replace cryptochromes in the mammalian oscillator and might be doing so using different mechanistic approaches, that also differ from the one used by mammalian cryptochromes (Kume et al., 1999; Shearman et al., 2000; reviewed by Reppert and Weaver, 2002).

In order to shed light on the mode of action of both CPD photolyases, *Pt*CPD-PL and Cc-PHR2, we studied in more detail the interaction between the photolyases and CLOCK, and how this interaction could affect CLOCK. It was previously shown that Cc-PHR2 and *Pt*CPD-PL can bind CLOCK, but not BMAL1, which results in inhibition of CLOCK/BMAL1-driven transcription and dampening of the oscillation of cultured fibroblasts (Biernat et al., 2012; Chaves et al., 2011). We have explored, hence, two possible action mechanisms by which Cc-PHR2 and *Pt*CPD-PL might work in the mammalian circadian clock. In the first scenario Cc-PHR2 and/or *Pt*CPD-PL may reduce binding of CLOCK to the DNA, thereby reducing the efficiency of transcription of E-box containing clock genes. A similar mechanism has been described for the *Drosophila* circadian clock where inhibition of CLOCK/CYCLE-driven transcription is achieved by PER and TIM repressors that inhibit binding of CLOCK/CYCLE to DNA (reviewed by Allada and Chung, 2010; Yu et al., 2006). According to the results obtained by the chromatin-immunoprecipitation experiment, this action mechanism is not likely as the amount of the CLOCK protein that binds chromatin was the same in the presence of either Cc-PHR2, *Pt*CPD-PL and mCRY1 (Figure 3, A). We cannot exclude, however, that CLOCK is overexpressed to such levels

that is located mainly in the cytoplasm. We know that co-expression of CLOCK and PHRs results in shifts of CLOCK to the nucleus (Biernat et al., 2012). We assume, therefore, that in chromatin-immunoprecipitation experiment the majority of CLOCK protein is localized in the nucleus. In order to be assured of this assumption, the control for localization of CLOCK should be still performed. The second scenario we have taken into account speculates that binding of Cc-PHR2 or *Pt*CPD-PL to CLOCK, might prevent CLOCK/BMAL1 heterodimer formation. Using a co-immunoprecipitation experiment, we showed that in the presence of either of these CPD photolyases, CLOCK/BMAL1 heterodimers are formed (Figure 3, B). According to both results, so far, there is no indication that the mechanism employed by these two CPD photolyases differs from the mechanism that is used by cryptochromes in the mammalian transcription-translation feedback loop. We postulate, therefore, that the tested class II CPD photolyases employ a mechanism similar to CRYs to act in the core oscillator.

In the mammalian circadian clock, CLOCK/BMAL1 heterodimers remain bound to E-box elements in the promoters over the circadian cycle. During transcriptional inhibition, the CRY-containing complex brings co-repressors to the promoter leading to inhibition of the CLOCK/BMAL1 heterodimer (Gekakis et al., 1998; Schearman et al., 2000; reviewed by Reppert and Weaver, 2002). The effect on the molecular oscillator is weaker for the class II CPD photolyases than for cryptochromes (Biernat et al., 2012; Chaves et al., 2011). Taking this information into consideration, we are tempted to assume that class II CPD photolyases might not be able, unlike mammalian cryptochromes, to interact and bring the co-repressor complex to the promoter. Probably, the whole mechanism that photolyases rely on to work in the TTFL is based on their ability to bind CLOCK protein. This interaction does not prevent CLOCK from binding chromatin or disrupts the CLOCK/BMAL1 complex, but may have influence on other processes that regulate transcription, such as the ability of CLOCK to mediate the acetylation of BMAL1 (Hirayama et al., 2007). Those aspects are highly interesting to pursue in order to further resolve the CPD photolyase way of action in the TTFL.

It is known that cryptochromes inhibit CLOCK/BMAL1-driven transcription by binding CLOCK (via the core domain of cryptochromes) and BMAL1 (via the predicted coiled-coil domain of cryptochromes). Previously, it was shown that removal of the complete C-terminal extension of CRY1 abolishes repressor activity towards CLOCK/BMAL1-driven transcription. Yet, specific deletion mutants in the CRY1 coiled-coil domain or its downstream tail domain did only weakly affect the inhibitory function of CRY1 (Chaves et al., 2011). The ability to bind CLOCK via the core domain and the interaction between the core domain and the C-terminal domain of CRY appears therefore mandatory for cryptochrome function, probably by providing the proper functional protein structure (Chaves et al., 2006; van der Schalie et al., 2007). Moreover, it was shown that not all photolyases possess a clock-like function in the mammalian circadian oscillator. *Arabidopsis thaliana* (6-4)PP-PL and Cc-PHR1 from ChchNPV, contrary to *Pt*CPD-PL and Cc-PHR2, did not have the ability to regulate the circadian clock (Chaves et al., 2011; Biernat et al., 2012). This finding is in line with the hypothesis that not amino acid sequence and domain content, but rather the overall protein structure that is acquired by the protein can result in clock function (Chaves et al., 2011).

Manipulation of insect host behaviour by baculoviruses, e.g. hypermobility and induced climbing behaviour, has been extensively observed (e.g. Goulson et al., 1997). The conserved viral ecdysteroid UDP glycosyltransferase (*egt*) gene was recently

identified to promote climbing behaviour (Hoover et al., 2011), while some viruses (but not ChchNPV) encode a protein tyrosine phosphatase (PTP) which leads to enhanced locomotion (Kamita et al., 2005; van Houte et al., 2011). Testing the circadian regulatory function of baculovirus photolyases in insect system, not mammalian, would truly reveal their function. Analyzing whether infection with a baculovirus encoding a dually functional photolyase affects the host insect's circadian clock, may reveal other forms of behavioural manipulation, for instance related to day/night feeding behaviour. To fully understand the aspect of the clock functional viral PHRs, PHRs null viruses need to be constructed and used as a negative control. It would be also interesting to study the molecular clocks in organisms possessing both a photolyase with a dual function and cryptochromes (marsupials). Testing other class II CPD photolyases, to trace the distribution of dual function within different organisms, would be valuable to establish when and why in the course of evolution some of the photolyases lost or kept duality of function.

In the present study we have shown that Cc-PHR2, similarly to *Pt*CPD-PL, can replace cryptochromes in the mammalian circadian clock. We have studied and discussed the possible molecular mechanisms behind the circadian action of dually-functional CPD photolyases and concluded that the overall structure of the protein, not its domain content, is probably necessary for its functionality as a clock protein. We suggest, therefore, that class II CPD photolyases, Cc-PHR2 and *Pt*CPD-PL, can act in the mammalian circadian clock in a mode comparable to cryptochromes.

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CHAPTER 7

General discussion

Baculoviruses are used as biocontrol agents of pest insects as they are highly virulent, host specific, environment-friendly and safe for not-target animals. Their effective use in the field is hampered, however, by the ultraviolet (UV) component of solar light, which induces cyclobutane pyrimidine dimers (CPDs) in (viral) DNA. CPD photolyases are enzymes that repair CPDs with the help of visible light, in a process called photoreactivation. It has been shown that the baculovirus *Chrysodeixis chalcites* nucleoplyhedrovirus (ChchNPV) possesses two photolyase genes, *Cc-phr1* and *Cc-phr2* (van Oers et al., 2004, 2005). Only *Cc-phr2* encodes a biochemically active photolyase that may protect this virus against UV irradiation (van Oers et al., 2005). CPD photolyases are members of the cryptochrome/photolyase family (CPF) of flavoproteins, which consist of two types of structurally conserved proteins that have different functions. Photolyases repair DNA, whereas cryptochromes work as photoreceptors or as core components of the circadian clock that drives 24 h rhythms in behavior, physiology, and metabolism. In mammals, cryptochromes act as inhibitors of CLOCK/BMAL1-driven transcription, and hence, regulate proper performance of the molecular oscillator that drives the circadian clock. A clock repression function has been reported for *Phaeodactylum tricornutum* (diatom) and *Ostreococcus tauri* (green alga) (6-4)PP photolyases (Coesel et al., 2009; Heijde et al., 2010). In this thesis, an attempt is made to determine the function, to study the evolutionary aspects, and investigate the ecological consequences of baculoviruses having CPD photolyases. The study described in this thesis focuses more specifically on: (i) the evolutionary path of baculovirus photolyases by analysing their relationship with other (putative) photolyase genes, (ii) the functional consequences of baculovirus photolyases by testing the UV sensitivity of ChchNPV occlusion bodies (OBs), and (iii) a regulatory function of baculovirus and other class II CPD photolyases on the circadian clock. In this chapter, all results assembled in this thesis will be discussed, summarising the current knowledge and suggesting directions for future research.

The complementation assay in a DNA repair-deficient *E. coli* strain performed previously pinpointed that Cc-PHR2, in contrast to Cc-PHR1, is active as a photolyase (van Oers et al., 2008). The *in vitro* assay performed simultaneously showed that Cc-PHR2 is able to restore thymine dimers to monomers, providing further evidence for the functionality of Cc-PHR2 as a CPD photolyase (van Oers et al., 2008). When the Cc-*phr2* gene was placed under the control of the *Orgyia pseudotsugata* (Op) MNPV immediate early 2 (*ie2*) promoter in the *Helicoverpa armigera* (Hear) NPV genome, photoreactivation and hence rescue of the biological activity of this recombinant virus was observed (Xu et al., 2010c, PhD thesis). However, this photolyase was not detected as a protein in ChchNPV ODVs upon LC-MS/MS analysis suggesting the contrary. The data obtained so far however, pointed into the direction that Cc-PHR2 may play a role in baculoviral DNA repair but at what point was not clear.

To address this issue bioassays were carried out with ChchNPV to investigate whether Cc-PHR1 and/or Cc-PHR2 make ChchNPV less sensitive to UV-light (Chapter 3). Viral OBs were UV-irradiated to inactivate the DNA in the occlusion derived form of the virus (ODV) and then exposed the OBs to visible light to allow photolyases to repair the CPD lesions. A second group of OBs was kept in the dark after UV-irradiation (no photoreactivation). The mortality observed was similar with both treatments, however, and independent of how long the photoreactivation was allowed to take place. It was therefore hypothesized that, at least in the case of ChchNPV, *phr1* and *phr2* are not active in OBs and this is in line with the observation that the products of these genes

(PHRs) are not present in ODVs (Xu et al., 2011).

It was hypothesized, so far, that the PHRs are packaged in ODVs and that this would eliminate the need for transcription and expression of PHRs after the virus entry, as well as the danger of transcribing mutated and/or nonfunctional *phr* genes (Xu et al., 2011). Moreover, such a hypothesis is also favored by the fact that the ODV, while persisting in the environment in the OB, is abundantly exposed to the visible light energy required for photoreactivation. The repair of UV-affected DNA would be more efficient, therefore, in such conditions. The data shown in Chapter 3, however, does not corroborate those assumptions. Moreover, PHR2-EGFP was not visible by confocal microscopy in OBs produced upon AcMNPV infection in *T. ni* High Five cells (Xu et al., 2010a). PHR2 and PHR2-EGFP were also not detected by 'western' analysis in OBs isolated from the same AcMNPV-infected cells (Xu, PhD thesis). The early putative motifs in the promoters of the *phr1* and *phr2* genes (CAGT and GATA for *phr1* and *phr2*, respectively; van Oers et al., 2004, 2005), the lack of PHRs in the ODVs by LC-MS/MS (Xu et al., 2011), the lack of PHR2 in OBs in confocal microscopy assay, when detecting PHR2 in OBs by western blotting (Xu, PhD thesis), and the lack of differences in UV-sensitivity of OBs from ChchNPV with or without photoreactivation (Chapter 3), suggest that PHRs might be expressed only in the early stages of infection. That might be a consequence of the fact that baculovirus *phr*s are most likely derived from an ancestral insect host (Chapter 2), and that *phrs,* probably together with their promoters, were transferred to the baculoviruses. It is not surprising, therefore, that photolyases are only present in the early stages of infection, and do not assemble in OBs. In order to establish the transcriptional profile of *phr1* and *phr2*, assays in the CcE-1 cell line (Xu et al., 2010b) in the presence or absence of protein and DNA synthesis e.g. using inhibitors could be performed to resolve this issue. Those assays would allow discriminating between immediate-early, delayed early and late genes. So far, it is assumed that photolyases are expressed early in the infection, as up till now the collected data are excluding the packaging of photolyases into ODVs and, so the late expression profile.

Another possibility is that the photolyases are only active when present as enzymes in the budded virus (BV). In the study on the activity of DNA repair enzyme cv-PDG, a pyrimidine dimer-specific glycosylase from green algal endosymbionts of *Paramecium bursaria* (van Etten and Meints, 1999; Furuta et al., 1997), it has been reported that when expressing cv-PSG in AcMNPV from the hsp70 promoter, the inactivation of the budded virus by exposure to UV light was 3-fold lower as compared to wild type AcMNPV (Petrik et al., 2003). OBs from such recombinants did not show, however, any difference in UV sensitivity when compared with wild type AcMNPV. Taking into consideration these data, it could be speculated that PHRs from ChchNPV are only active, if at all, in association with BVs, but not with ODVs. To prove this, ChchNPV BVs should be subjected to LC-MS/MS analysis. There is, however, no knowledge to what extent light penetrates into the insect midgut to facilitate photoreactivation. So, the possibility that PHRs act prior to infection as is the case for poxvirus PHRs (Srinivasan et al., 2001) seems remote.

At least in the case of Cc-PHR2, the photolyase thus might not only play a role in DNA repair as a consequence of UV inactivation. Another possibility is that photolyases, as proteins involved in DNA damage responses, are involved in the replication of the virus in the insect host cells. It was shown, recently, that baculoviral infections induce a DNA damage response that is required for efficient viral replication (Huang et al.,

2011). It was abundantly shown that many viruses use their ability to manipulate the DNA damage responses and associated downstream pathways in order to benefit during their replication process (reviewed by Chaurushiya et al., 2009). Some of the mammalian viruses are able to activate the DNA damage checkpoint proteins ATM (ataxia telangiectasia mutated; activated mainly in response to double-stranded DNA breaks) and ATR (ATM- and rad-3-related; activated mainly in response to single-stranded DNA breaks) signaling, which are crucial for their replication (Pickering et al., 2011; Lau et al., 2005; Lilley et al., 2005). The serine/threonine kinases ATM and ATR activate checkpoint signaling by phosphorylating substrates (e.g. CHK1, P53) at SQ/TQ motifs. AcMNPV-infected cells contain fragments of viral DNA that are thought to be replication intermediates and might mimic double- and single-stranded breaks (Mikhailov et al., 2009). Such replication intermediates are interpreted by the cell as damaged DNA and serve as triggers for the DNA damage response. It was further shown that by inhibiting ATM and ATR signaling, AcMNPV DNA replication was decreased (Huang et al., 2011). It is suggested, furthermore, that the virus is utilizing factors that are activated by the DNA damage response to fully achieve the high levels of DNA replication (and thus the late and very late gene expression), which depend on DNA replication. Although the identities of those factors remain still to be determined, it is a possibility that they may be involved in DNA repair or recombination (Huang et al., 2011). Taking the above observations into consideration, it is appealing to speculate that ChchNPV replication would also be DNA damage response-dependent and thus ChchNPV would induce such a response. It is concluded therefore, that photolyases as DNA repair proteins could contribute to those processes and be involved in boosting up the DNA replication and late and very late gene expression. Moreover, the presence of Cc-PHRs in the virogenic stroma (Xu et al., 2010a) and the fact that *phrs* are early expressed genes further support the hypothesis that photolyases may contribute to replication processes.

Why do some baculoviruses have *phr* genes (Chapter 2) and others not? AcMNPV and other MNPVs have multiple nucleocapsids and hence genome copies per ODV. These genome copies can recombine prior to DNA replication and viable virions can be produced, hence no need for DNA photolyases. ChchNPV is a single (S) capsid nucleopolyhedrovirus and has less opportunity to use recombination to repair DNA damage. That means that ChchNPV, possessing only one genome copy per ODV, would be more susceptible to mutagenic processes. All baculoviral *phrs* identified so far, were found in SNPVs or GVs (Chapter 2). SpliGV (or a direct ancestor) was the first baculovirus to obtain a *phr* gene from its lepidopteran host, which was subsequently horizontally transferred to other group II NPVs, including the plusiine-infecting group II NPVs like ChchNPV (Chapter 2). This could be a consequence of the fact that viruses that contain only a single genome copy per virion are more susceptible to UV-mediated inactivation. Additionally, in contrast to MNPVs that contain multiple genome copies, neither SNPVs nor GVs can benefit from additional recombination and complementation events in the host cell. Most likely, SNPVs and GVs need different mechanisms to control DNA damage and to facilitate proper replication.

In this case presence of photolyase genes in SNPVs and/or GVs would give the SNPVs advantage and DNA repair abilities (in the early stages of infection or in a budded form of the virus), which are needed when the virus cannot contribute from multiply genome copy. This assumption is supported by the earlier mentioned study by Petrik et al., (2003), which shows that the DNA repair enzyme cv-PDG is able to protect the

budded form of the virus against UV inactivation. Importantly, the expression of cv-PDG also increased the virulence of AcMNPV when fed to neonate *S. frugiperda* larvae, irrespectively of UV light conditions. The virus dose required for lethality was 16-fold lower, whereas the time needed for larval death was decreased by almost 50% in comparison to wild type AcMNPV (Petrik et al., 2003). It was suggested that the expression of cv-PDG has toxic effects on the midgut epithelial cells of *S. frugiperda.* The results might also suggest, however, that the possession of the DNA repair protein would accelerate the replication and infection process by participating in the DNA damage response pathways mentioned earlier. To fully understand the relationship between DNA damage response and viral infectivity and the role of class II CPD photolyases and their DNA repair capacity therein, PHRs null viruses need to be used as a negative control. A photolyase knockout ChchNPVs can be generated after constructing a ChchNPV bacmid in which photolyase gene is inactivated by insertion of a bacterial cassette. Alternatively, RNAi technology can be exploited in knockdown assays and it can be tested whether down-regulation of *phrs* is enough to alter the replication parameters of the virus. These approaches will allow us to assess whether the replication of virus is affected by the presence of photolyase and which pathways are triggered by this repair enzyme. The fact that also many SNPVs and GVs lack photolyases and that their genomes are relatively stable when passaged many times in insects makes the function of baculovirus photolyases in UV damage repair an unlikely possibility.

Many pathogens manipulate the behavior of their hosts in order to enhance transmission of the progeny pathogens to the new host (zombie ants, Andersen et al., 2009). That phenomenon is as well known for baculoviruses that induce changes in behavior in their caterpillar hosts, such as tree-top disease (Goulson et al., 1997). The baculoviral gene ecdysteroid UDP glycosyltransferase (*egt*) was recently shown to enhance climbing behavior of its host (Hoover et al., 2011). Some viruses (but not ChchNPV) encode a protein tyrosine phosphatase (PTP), which leads to enhanced locomotion (Kamita et al., 2005; van Houte et al., in preparation). CPD photolyases, as homologs of mammalian cryptochromes, might impinge on circadian performance. In Chapters 4, 5 and 6 the focus was on the functional evolution of cryptochromes and photolyases, members of cryptochrome/photolyase family of flavoproteins. Cryptochromes, unlike photolyases, are not involved in DNA repair (Lin and Todo, 2005). Rather, they are responsible for completely different, light-mediated processes. Cryptochromes act as blue-light photoreceptors in plant and act as regulators of the circadian clock in mammals (Cachmore et al., 2003; van der Horst et al., 1999). In mammals cryptochromes are a part of the circadian clock that drives daily rhythms in behavior, physiology and metabolism. Mammalian cryptochromes (CRY1 and CRY2) act in the molecular oscillator as repressors of the CLOCK/BMAL1 transcription activation complex by physically interacting with CLOCK (via the CRY core domain) and BMAL1 (via the predicted coiled-coil domain in the C-terminal part of the CRY proteins). In Chapters 4 and 6, it was shown that Cc-PHR2 (in contrast to Cc-PHR1) can mimic mammalian cryptochromes and even can revive the molecular clock in arrhythmic *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ (Cry double knockout) cells and tissues. In Chapter 5, two other photolyases (i.e. *Potorous tridactylus* CPD-PL from the same class II CPD photolyases and *Arabidopsis thaliana* (6-4)PP-PL from (6-4)PP class of photolyases) were studied in terms of their functionality in the mammalian circadian clock. It was shown that, whereas *At*(6-4)PP-PL does not impinge on the circadian clock, *Pt*CPD-PL affects the performance of the circadian clock and is able to reconstitute the clock in a

Cry1$^{-/-}$*/Cry2*$^{-/-}$ background in the same way as Cc-PHR2. Cc-PHR2 and *Pt*CPD-PL can bind CLOCK but not BMAL1, which result in inhibition of CLOCK/BMAL1-driven transcription and dampening of the oscillation of cultured fibroblasts (Chapter 4 and 5).

It was therefore hypothesized, therefore, that Cc-PHR2 might have an impact on the behavior of the infected host by influencing its circadian clock. For baculoviruses it might be highly advantageous to influence the circadian clock of its host in order to facilitate a safer and more efficient spreading of the viral progeny. For instance, one can speculate that photolyase-mediated changes in the host circadian system may shift climbing behavior towards the night. As a consequence, spread of progeny ODVs would not be affected by UV light (as shown, photolyase does not protect the ODVs against the UV light, at least in case of ChchNPV; Chapter 3). The principle of a parasite affecting on the circadian clock of its host is not unprecedented. The studies with *D. melanogaster* have shown that circadian rhythms were lost due to infection of the fly with *Streptococcus pneumonia* or *Listeria monocytogenes* (Shirasu-Hiza et al., 2007). The sick flies displayed fewer sleep bouts and shorter sessions of continuous sleep than healthy flies. Moreover, circadian mutant flies (*period* and *timeless* mutants) were more sensitive to the infection with these bacteria (Shirasu-Hiza et al., 2007). In a similar fashion it is thus possible that, by regulating the circadian rhythms of the insect host, virus would enhance its pathogenicity.

Another possible scenario is that photolyases might have an effect on the feeding behavior of their hosts. The circadian clock and metabolism are tightly linked (reviewed by Huang et al., 2011). In *D. melanogaster*, the clock-regulated output gene *takeout* (*to*) is responsible for the regulation of feeding behavior under starvation (Meunier et al., 2007; Sarov-Blat et al., 2000). Under starvation, expression of *to* appeared upregulated, which resulted in the increase of the locomotor activity. It is tempting to speculate, therefore, that photolyases could also have an effect on the feeding pattern of the virus-infected insects. By manipulating the host circadian clock to increase foraging, the virus would benefit from a bigger mass of the insect as this translates into higher progeny virus yield. That could affect also molting comparably to the effects that are described for *egt*. Moreover, analyzing whether infection with baculovirus encoding a dually functional photolyase affects the insect's circadian clock may reveal other forms of behavioral manipulation, for instance related to day/night feeding behavior. Therefore, comparing healthy versus infected insects' circadian behavior patterns, feeding patterns and locomotor activity, could reveal whether the infection by such a photolyase-containing virus would as well result in changes of the insect host behavior. To fully reveal the impact of photolyases from baculoviruses on the behavior of their host the Cc-*phr* null virus, serving as a negative control, must be available. Importantly, as the circadian regulatory function of baculovirus photolyases was tested in mammalian cells (Chapter 4), these studies should be extended to the insect system in order to truly reveal their function. In this context, it will be interesting to examine the effects of photolyases on circadian gene expression of the virus-infected insects as the outcome of such survey would help to understand the mechanisms behind the circadian functionality of photolyases.

The same can hold for other organisms that contain photolyases, or both photolyases and cryptochromes. Testing the molecular basis of the circadian clock of those organisms could provide an explanation for the function of photolyases in those systems and their relationship with other clock proteins. Therefore, the effect of the

down-regulation of *Pt*CPD-PL was studied in *Potorous tridactylus* kidney cells (*Pt*K2) stably expressing mPer2::luc (Figure 1). siRNA-mediated downregulation of PtCPD-PL managed to affect circadian oscillations in PtK2 cells by shifting the phase of oscillations (Figure 1). The *Pt*K2 cell line is stably expressing expressing a luciferase reporter gene driven by the mPer2 promoter. The effect of down-regulation of the *Pt*CPD-PL by use of siRNA directed against PtCPD-PL was studied by measuring the bioluminescence rhythms of forskolin-synchronized PtK2 cells. Figure 1 shows that by down-regulating photolyase, the phase of the circadian oscillation is altered. This observation, together with data presented in Chapter 5, is highly relevant as it shows that indeed the Potorous circadian clock is adapted to the presence of the CPD photolyase and might be involved in regulating the circadian oscillator. These data, by suggesting the circadian role of *Pt*CPD-PL, encourage studying further photolyases in the context of the organism that they originate from to resolve the mechanism of dually functional CPD photolyases.

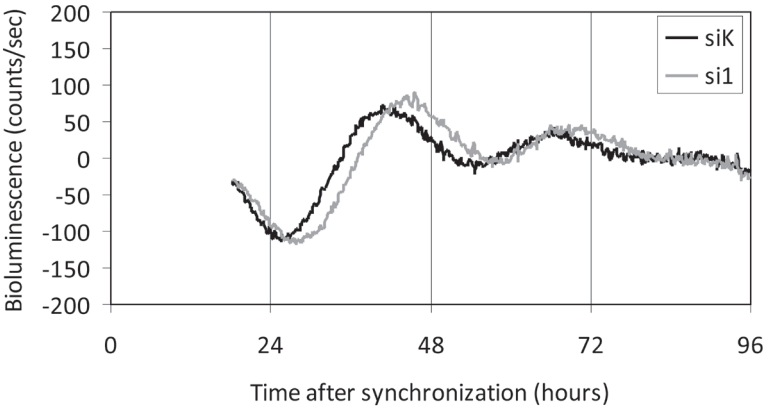

Figure 1. Representative example of bioluminescence rhythms of forskolin synchronized *Potorous tridactylus* kidney cells (*Pt*K2)-mPer2::luc after transient transfection with *Pt*CPD-PL siRNAs (si1) or control siRNA (siK).

In Chapter 6, an attempt was made to clarify the molecular mechanism underlying the circadian clock regulatory function of Cc-PHR2 and *Pt*CPD-PL by investigating the CLOCK binding ability of both photolyases. Three different mechanisms are envisaged by which binding of photolyases to CLOCK might affect the circadian oscillator. In the first two options, photolyases were hypothesized to either prevent CLOCK to bind the E-box elements in promoters or to prevent the formation of CLOCK/BMAL1 heterodimer, thus resulting in decreased transcription activation of E-box genes by the CLOCK/BMAL1 heterodimer. The chromatin- and co-immunoprecipitation experiments do not support such mode of action. It was assumed, therefore, that the mechanism used by class II CPD photolyases resembles the mechanism used by mammalian cryptochromes, where CLOCK/BMAL1 heterodimers remain bound to E-box elements in the promoters over the circadian cycle and during transcription inhibition, whereas the CRY-containing complex brings co-repressors to the promoter, leading to inhibition of the CLOCK/BMAL1 heterodimer (Reppert and Weaver, 2002). Taking that information into consideration, it is assumed that class II CPD photolyases might associate with DNA bound CLOCK/BMAL1 and inhibits transcription activation. However, unlike mammalian cryptochromes, may not interact with, and bring co-repressor proteins to the promoter, which may explain

why the effect of class II CPD photolyases on the molecular oscillator is weaker than the effect of cryptochromes (Chapter 4 and 5). Probably, the ability of photolyases to affect the transcription-translation feedback loop (TTFL) in the molecular oscillator is based on their ability to bind CLOCK protein. Moreover, taking into account the lack of clock function of *At*(6-4)PP-PL and Cc-PHR1, it is highly possible that not the amino acid sequence or the domain content, but rather the overall structure that is adopted by the protein, can result in clock function, and thus, there is no need to implement a different mechanism. Although the interaction between CLOCK and PHRs does not prevent CLOCK from binding chromatin or disrupt the CLOCK/BMAL1 complex, but may have influence on other processes that regulate transcription. As such, it will be important to investigate potential differences in the post-translational modification of clock proteins in presence of photolyases. Moreover, testing the expression levels of other clock genes in presence or absence of photolyase might establish how and why photolyases, although different from cryptochromes, are able to play a similar role in the circadian clock.

The crystal structure of a class II CPD photolyase, in free state as well as in complex with CPD lesion-containing duplex DNA, has been recently resolved (Kiontе et al., 2011). Crystallized *Methanosarcina mazei* Mm0852 is a class II photolyase of the archeal order Methanosarcinales. Mm0852 is closely related to homologs from plants (Iwamatsu et al., 2008) and from animals, like the marsupial *Potorous tridactylus* (Yasui et al., 1994). Therefore, the crystallized enzyme structure can be easily transferred to all other metazoan photolyases. That analysis revealed many differences between class I and class II photolyases, like a different lesion binding mode and usage of a conserved tryptophane dyad (not triad like in the class I CPD photolyases) as electron transfer pathway to the catalytic FAD cofactor. The absence of any bound chromophore antenna for Mm0852 was also reported, which could be connected however to the expression system used. This knowledge on the core domain structure will facilitate designing mutations in class II CPD photolyases in order to see which domains are responsible for regulating the circadian clock. It would be possible to pinpoint the domains in both cryptochromes and photolyases, which are crucial for the clock function. Additionally, it would facilitate to clarify the molecular mechanisms that determine the functionality of photolyases in the TTFL.

In this PhD thesis, the potentially dual function of class II photolyases was investigated. PHRs from ChchNPV, as DNA repair proteins, might be also involved in DNA damage responses, which can bring the replication advantage for the virus during infection and life cycle in the host cell. It is as well possible that the CPD photolyases might possess a circadian clock regulatory function. It would be highly advantageous for the baculovirus to be able to control the circadian clock of their infected host and thus being able to alter its feeding patter and locomotor activity, which could translate in e.g. higher virus yield and transmission rates. Those aspects are very interesting to test further in order to reveal the true function(s) of (baculoviral) CPD photolyases.

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List of abbreviations

AAV	Adeno-associated virus
At(6-4)PP-PL	*Arabidopsis thaliana* Pyrimidine–pyrimidone (6-4) photoproduct photolyase
8-HDF	8-hydroxy-7,8-didemethyl-5-deazaflavin
AMEV	*Amsacta moorei* entomopoxvirus
BCA	Bicinchoninic acid
BER	Base excision repair
BEVS	Baculovirus expression vector system
bHLH-PAS	basic-helix–loop-helix PER-ARNT-SIM transcription factor
BMAL1	aryl hydrocarbon receptor nuclear translocator-like, also known as ARNTL, or MOP3
bp	Base pair
BSA	Bovine serum albumin
BV	Budded virus
Cc-PHR1	ChchNPV encoded photolyase 1
Cc-PHR2	ChchNPV encoded photolyase 2
cDNA	copy Deoxyribonucleic acid
CLOCK	circadian locomotor output cycles kaput
CPD	Cyclobutane pyrimidine dimer
CRY	Cryptochrome
Cry-DASH	Cryptochrome - *Drosophila*, *Arabidopsis*, *Synechocystis*, human
CSF	Classical swine fever
EGFP	Enhanced green fluorescent protein
EGT	Ecdysteroid UDP-glucosyltransferase
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FMN	Flavin mononucleotide
FWPV	Fowlpox virus
GT	Guatemala
GV	Granulovirus
ie2	Immediate early 2 gene
IgG	Immunoglobulin G
IP	Immunoprecipitaion
LD	Light dark
LC-MS/MS	Liquid chromatography-linked tandem mass spectrometry
mCRY	mammalian CRYptochrome
MNPV	Multiple nucleopolyhedrovirus
MOI	Multiplicity of infection
MSEV	Melanoplus sanguinipes entomopoxvirus
MTHF	5,10-methenyltetrahydrofolate
MW	Molecular weight markers
MYXV	Myxomavirus
NCBI	National Center for Biotechnology Information
NER	Nucleotide excision repair
NJ	Neighbor-joining
NL	Netherlands/Dutch
NLS	Nuclear Localisation Sequence
NPV	Nucleopolyhedrovirus
OB	Occlusion body
ODV	Occlusion-derived virus
ORF	Open reading frame
p.i.	Post infection
p.t.	Post transfection
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PEP	Polyhedrin envelope protein
PER	Period
phr	DNA photolyase gene
PIF	Per os infectivity factor
Polh	Polyhedrin gene
PtCPD-PL	Potorous tridactylus encoded cyclobutane pyrimidine dimer photolyase
qPCR	Quantitative real-time PCR
REV-ERBα	reverse of ErbA alpha
RORα	RAR-related orphan receptor-α
RT-PCR	Reverse-transcriptase polymerase chain reaction
SCN	suprachiasmatic nucleus
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFV	Shope fibroma virus
SNPV	Single nucleopolyhedrovirus
SP	Spanish
TIM	Timeless
UV	Ultraviolet
VLP	Virus-like particle
(6-4)PP	Pyrimidine–pyrimidone (6-4) photoproduct

Summary

The family *Baculoviridae* consists of large, enveloped DNA viruses, which infect invertebrates, mainly insects from the order Lepidoptera. These viruses are highly virulent, often host specific and environmentally safe. Hence, they are considered as an attractive alternative to chemical pesticides in insect pest control. For this purpose, viral occlusion bodies (OBs) containing baculovirus virions are sprayed over the foliage. One of the main factors that limit the effective performance of baculoviruses in the field is their susceptibility to inactivation by solar ultraviolet (UV) light.

UV light destructively affects DNA by inducing cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone (6-4) photoproducts (6-4PPs) that cause distortions in the DNA. In order to repair CPDs and (6-4)PPs almost all organisms (except placental mammals, which are using different mechanisms) are equipped with photolyases, CPD photolyases and (6-4) photolyases. Photolyases are lesion-specific and need visible light to carry out their catalytic function (photoreactivation). Due to their conserved structure, photolyases are part of a family of flavoproteins, the cryptochrome/photolyases family (CPF). Members of this family have various functions, directly and/or indirectly responding to light. Whereas photolyases are UV-damaged DNA repair enzymes, other members of the CPF, cryptochromes, are involved in photoreception and/or function as core components in the circadian clock.

CPD photolyases are further divided into two classes, based on sequence divergence. Class II CPD photolyases are conserved in a specific clade of baculoviruses that infect insects of the Plusiinae subfamily of the family Noctuidae. *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV), which infects the tomato looper *C. chalcites,* possesses two photolylase genes, named *Cc-phr1* and *Cc-phr2*. Cc-PHR2, in contrast to Cc-PHR1, has been shown to act as a functional photolyase in a heterologous system. Most baculoviruses, however, do not contain *phr* genes. It also has been shown that both Cc-PHRs are targeted to the insect cell nucleus and associate with DNA. In addition, Cc-PHR2 co-localized with the virogenic stroma, the site of viral replication. The study described in this thesis aimed at a better understanding of the functional and evolutionary aspects of (baculovirus) CPD photolyases.

In Chapter 2, a phylogenetic study was performed to trace the evolutionary path of baculovirus photolyases by analysing the relationship with other photolyase genes. To that aim, partial *phr* cDNA sequences derived from the plusiine insects *C. chalcites* and *T. ni*, and from *S. exigua* (another noctuid) were characterised and subsequently used for the phylogenetic analysis. A *polyhedrin/granulin* gene phylogeny was constructed to facilitate the interpretation of the photolyase phylogeny. It was concluded that *phr* genes from lepidopteran insects are highly related to the baculovirus homologues, while the baculovirus *phr* genes are more related to each other than to the photolyases of their host insects. We have postulated, therefore, that a CPD photolyase gene was introduced into the baculoviruses by a single horizontal gene transfer from a lepidopteran insect into an ancestral baculovirus.

Cc-PHR2, but not Cc-PHR1, has been shown to contain photoreactivating activity, and as such can repair DNA lesions. Yet, it was not known whether these PHRs are involved in protection of ChchNPV against UV light. Therefore, in Chapter 3, the UV sensitivity of ChchNPV occlusion bodies (OBs) was investigated. Bioassays were developed to measure the effect of UV irradiation (with or without subsequent

exposure to photoreactivation light) on the mortality induced by ChchNPV in *C. chalcites* larvae. We observed that the mortality of test larvae did not increase when OBs were photoreactivated. This is in line with the absence of photolyases in the occluded virions present in OBs, as concluded from proteomic analysis of these virions performed in a parallel study. Photolyases are probably expressed only in the early phases of infection and only then may repair DNA or perform other functions.

Photolyases are homologous to cryptochromes, core regulators in the mammalian circadian clock. Cryptochromes act in the molecular oscillator as repressors of the CLOCK/BMAL1 complex by interacting with CLOCK via their core domain and with BMAL1 via a predicted C-terminal coiled-coil domain. Therefore, based on the sequence homology between cryptochromes and the baculovirus photolyases and the lack of photolyase activity of Cc-PHR1 in complementation assays, we explored the possibility that these CPD photolyases play a role in the molecular circadian oscillator, similar to cryptochromes. In Chapter 4, the potential interaction of Cc-PHR1 and Cc-PHR2 with core elements of the circadian oscillator (i.e. CLOCK and BMAL1) was studied. Co-immunoprecipitation and immunofluorescence experiments showed that both CPD photolyases can physically interact with the CLOCK protein, but not with BMAL1. Subsequently, luciferase assays were performed in order to test whether this physical interaction would translate into a physiological response. Only Cc-PHR2 inhibited CLOCK/BMAL1-driven transcription and thus acted as a cryptochrome. It was also shown that overexpressed Cc-PHR2 (but not Cc-PHR1) decreased the amplitude of the oscillation of cultured mouse dermal fibroblasts (MDFs), which is likely due to its capacity to efficiently inhibit CLOCK/BMAL1-driven transcription. It is known that upon baculovirus infections the insects' behaviour is highly modified, which is exhibited by for instance hypermobility and climbing. It is appealing to speculate that the presence of a photolyase with a potential clock regulatory function could bring an extra ecological benefit for baculoviruses by playing a role in virus-induced behavioural changes through the host circadian clock.

Two other photolyases, the class II CPD photolyase from *Potorous tridactylus* (*Pt*CPD-PL) and the (6-4)PP photolyase from *Arabidopsis thaliana* (*At*(6-4)PP-PL), which are distantly and closely related to CRY1, respectively, were also examined for a clock regulatory function. In Chapter 5, transgenic mice with a *β-actin* promoter-driven *At*(6-4)PP-PL or *Pt*CPD-PL gene were used to demonstrate that the presence of *Pt*CPD-PL, in contrast to At(6-4)PP-PL, was affecting the circadian clock by shortening the period length of circadian behaviour. *Pt*CPD-PL, like Cc-PHR2, when overexpressed in cultured MDFs, was able to decrease the amplitude of the circadian oscillation, probably due to the observed interaction between the *Pt*CPD-PL and CLOCK proteins. The observation that *Pt*CPD-PL also inhibited CLOCK/BMAL1-driven transcription proved that also this CPD photolyase can play a regulatory role in the mammalian circadian clock. *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ double-knockout MDFs were used to further study the capacity of *Pt*CPD-PL to act as a true cryptochrome. To that aim, *Pt*CPD-PL and CRY1 were stably expressed from the *Cry1* promoter, in arrhythmic *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ MDFs. The presence of either *Cry1* or *Pt*CPD-PL restored the rhythms of this Cry-deficient cell line. Moreover, *Pt*CPD-PL was able to restore the circadian oscillator in the liver of (clock-deficient) *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice, demonstrating that *Pt*CPD-PL can act as a genuine cryptochrome. These findings shed new light on the importance of the core structure of mammalian cryptochromes in relation to its function in the circadian clock, and as such contribute to our understanding

of the evolution of the cryptochrome/photolyase family.

In Chapter 6, a more in-depth analysis of Cc-PHR2 and *Pt*CPD-PL was performed. Like *Pt*CPD-PL, Cc-PHR2 was shown to restore rhythmicity of a CRY-less mutant mammalian oscillator, both in cell culture and in mice. Bioluminescence assays with *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ fibroblasts, stably expressing Cc-PHR2 from the *Cry1* promoter, indicated that this photolyase can revive the circadian clock in arrhythmic *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ MDFs, as well as in the liver of Cry-deficient mice. Next, we investigated the molecular mechanism that could be implemented by Class II CPD photolyases to act in the circadian oscillator. Chromatin immunoprecipitation experiments showed that the presence of Cc-PHR2 and/or *Pt*CPD-PL does not prevent transcription of CLOCK/BMAL1 by affecting the binding of CLOCK to chromatin, i.e. to E-box elements in the DNA. Moreover, a co-immunoprecipitation assay demonstrated that Cc-PHR2 and/or *Pt*CPD-PL did not inhibit CLOCK/BMAL1 heterodimer formation. Therefore, photolyases are not competing with BMAL1 for binding to the CLOCK protein. These data suggest that the studied class II CPD photolyases, Cc-PHR2 and *Pt*CPD-PL act in the mammalian circadian clock, likely through a similar molecular mechanism as the mammalian cryptochromes. This observations lead to the conclusion that the overall structure of the protein, rather than the amino acid sequence or domain content, determines whether a functional interaction occurs between the CLOCK/BMAL1 heterodimer and the CPF member.

In conclusion, an ancestral CPD photolyase gene was most likely introduced once into the family *Baculoviridae* by a horizontal gene transfer from a lepidopteran insect, as the *phr* genes in this order of insects are highly related to the baculovirus homologues. Despite the fact that ChchNPV encodes a biochemically active photolyase, it is not able to restore infectivity of OBs after UV inactivation. In addition, it is shown that class II CPD photolyases not only repair UV damage in DNA, but also can interfere with (mammalian) clock function. These finding lead to new insights into the functional evolution of CPF members in general, and may give clues for possible ways of host modulation by photolyases-encoding baculoviruses.

Samenvatting

De familie *Baculoviridae* bestaat uit grote DNA virussen met een lipidenmembraan. Deze virussen infecteren ongewervelde dieren en komen vooral voor in insecten van de orde Lepidoptera. De virussen zijn dodelijk voor insecten, meestal voor specifieke soorten en daardoor veilig voor de omgeving. Daarom worden ze beschouwd als een aantrekkelijk alternatief voor chemische middelen ter bestrijding van plaaginsecten. Voor deze toepassing wordt het gebladerte van het te beschermen gewas besproeid met baculovirussen in de vorm van virale insluitlichamen (polyeders). Een belangrijke factor die het effectief toepassen van baculovirussen als pesticiden in de weg staat, is hun gevoeligheid voor de ultraviolette (UV) component van het zonnespectrum.

UV-licht beschadigt DNA, onder andere doordat het cyclobutaan-pyrimidine-dimeren (CPD) en pyrimidine-pyrimidone (6-4) fotoproducten (6-4PP's) induceert, wat leidt tot veranderingen in de DNA-structuur. Om de geïnduceerde CPD's en (6-4)PP's weer te verwijderen zijn bijna alle organismen (behalve de placentazoogdieren, die andere mechanismen gebruiken) toegerust met fotolyases. Deze enzymen zijn specifiek voor CPD's of (6-4)PP's en hebben zichtbaar licht als energiebron nodig om hun katalytische functie uit te kunnen oefenen (fotoreactivering). Op basis van hun geconserveerde structuur maken fotolyases deel uit van een familie van flavoproteïnen, de cryptochroom/fotolyasefamilie (CFF). De leden van deze familie hebben verschillende functies in de cel en reageren direct of indirect op licht. Terwijl fotolyases enzymen zijn die UV-schade aan DNA repareren, zijn andere leden van de familie, de cryptochromen, betrokken bij lichtreceptie en/of fungeren als cruciale onderdelen van de biologische of circadiane klok (dag/nacht ritme).

CPD-fotolyases zijn onderverdeeld in twee klassen, gebaseerd op de mate van genetische verwantschap. Klasse II CPD-fotolyases zijn aangetroffen in een bepaalde cluster van baculovirussen, namelijk die welke voorkomen bij insecten in de onderfamilie Plusiinae van de de familie Noctuidae. Een voorbeeld is *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV), dat infectieus is voor de Turkse mot *C. chalcites.* De meeste andere baculovirussen coderen niet voor fotolyase (*phr)* eiwitten. ChchNPV heeft twee fotolylase genen, genaamd *Cc-phr1* en *Cc-phr2*. Voor het CC-PHR2 eiwit, maar niet voor CC-PHR1, is in een heteroloog systeem aangetoond dat het fotolyaseactiviteit bezit en DNA schade kan repareren. Voor beide Cc-PHR's is aangetoond, dat ze in de celkern aanwezig zijn en daar binden aan DNA. Cc-PHR2 werd ook aangetroffen in het virogeen stroma, de plek waar virale DNA-replicatie plaatsvindt. De studie, als beschreven in dit proefschrift, had tot doel om de functiionele en evolutionaire aspecten van (baculovirus) fotolyases.

In Hoofdstuk 2 werd een fylogenetische studie uitgevoerd om de verwantschap van baculovirusfotolyases met andere fotolyases te onderzoeken en zo hun evolutionaire pad te herleiden. Hiertoe werden (gedeeltelijke) *phr* cDNA- sequenties opgehelderd van de motten *C. chalcites* en *Trichoplusia ni* (beide behorend tot de Plusiinae), en van *Spodoptera exigua,* behorende tot een andere onderfamilie van de Noctuidae. Deze cDNA sequenties werden vervolgens gebruikt voor fylogenetische analyses. Daarnaast werd een *polyhedrine/granuline* genfylogenie opgezet om de interpretatie van de fotolyasefylogeny mogelijk te maken. De conclusie was dat *phr*-genen van insecten van de orde Lepidoptera in hoge mate verwant zijn aan die van baculovirussen. Verder bleek de verwantschap tussen de baculovirus *phr*-genen onderling groter dan tussen de individuele

baculovirus *phr*-genen en die van de bijbehorende gastheer. Daarom is gepostuleerd dat CPD fotolyases in de baculovirusfamilie zijn terecht gekomen door een enkelvoudige, horizontale genoverdracht van een mot naar een voorouderlijk baculovirus.

Cc-PHR2 is in staat om DNA-laesies te herstellen, maar voor Cc-PHR1 is een dergelijke fotoreactiveringsactiviteit niet aangetoond. Het was echter niet bekend of deze PHRs het virus ChchNPV ook beschermen tegen UV-licht. In Hoofdstuk 3 werd daarom de UV-gevoeligheid van het virus geanalyseerd. Biologische experimenten werden opgezet om het effect van UV-bestraling te meten op de mortaliteit van ChchNPV voor *C. chalcites* rupsen. Bij deze proeven werd het virus in de vorm van polyeders bestraald met UV-licht en deze behandeling werd al dan niet gevolgd door belichting met fotoreactiverend, zichtbaar licht. De sterfte onder de larven nam niet toe door een fotoreactiveringsbehandeling van het virus. Hieruit kan worden afgeleid dat het fotolyase niet actief is vóór de infectie om DNA schade te herstellen. Inmiddels is uit een parallelle proteoomstudie gebleken dat de geteste virusdeeltjes ook geen fotolyase-eiwit bevatten, hetgeen de resultaten van deze biotoetsen verklaard. Fotolyases zijn dus waarschijnlijk alleen vroeg in de infectie aanwezig in cellen en alleen dan zouden ze DNA kunnen repareren, dan wel een andere rol kunnen spelen.

Fotolyases zijn homoloog aan cryptochromen, centrale regulators in de circadiane klok van zoogdieren. Cryptochromen fungeren in de 'moleculaire oscillator' als remmers van het CLOCK/BMAL1-eiwitcomplex door een interactie aan te gaan met CLOCK-eiwitten via hun centrale domein en met BMAL1 door middel van een voorspeld C-terminaal 'coiled-coil' domein. Gezien de sequentieovereenkomsten tussen cryptochromen en baculovirus CPD-fotolyases én het ontbreken van fotolyaseactiviteit in Cc-PHR1, werd onderzocht of deze fotolyases, vergelijkbaar met cryptochromen, een rol kunnen spelen in de moleculaire circadiane oscillator. In Hoofdstuk 4 is daarom gekeken naar een mogelijke interactie van Cc-PHR1 en Cc-PHR2 met hoofdelementen van de circadiane oscillator (te weten CLOCK en BMAL1). Co-immunoprecipitatie- en immunofluorescentie-experimenten lieten zien dat beide CPD-fotolyases een fysieke interactie kunnen aangaan met het CLOCK-eiwit, maar niet met BMAL1. Vervolgens werden toetsen uitgevoerd in een zoogdiersysteem en met een 'reporter' gen (luciferase) om te bepalen of deze fysieke interactie zich kan vertalen in een fysiologische reactie. Alleen Cc-PHR2 remde CLOCK/BMAL1-aangedreven transcriptie en werkte in dit systeem dus als een cryptochroom. Het bleek ook dat overexpressie van Cc-PHR2 (maar niet van Cc-PHR1) de amplitude (uitslag) van de oscillatie van gekweekte dermale muizenfibroblasten (MDFs) verminderde, wat waarschijnlijk het gevolg is van het vermogen van Cc-PHR2 om de CLOCK/BMAL1-aangedreven transcriptie efficiënt te blokkeren. Het is bekend dat als gevolg van een baculovirusinfectie het gedrag van de besmette insectenlarven wordt beïnvloed. Dit uit zich o.a. in hypermobiliteit en klimgedrag. Het is verleidelijk om te speculeren dat de aanwezigheid van een fotolyase met een mogelijk regulerende functie in de biologische klok het baculovirus ecologisch voordeel zou kunnen verschaffen. Hierbij kan men denken aan gedragsaanpassingen, geinduceerd in de gastheer via het moduleren van de circadiane ritme.

Twee andere fotolyase-eiwitten, het klasse II CPD fotolyase van *Potorous tridactylus* (*Pt*CPD-PL), en het (6-4)PP fotolyase van *Arabidopsis thaliana* (*At*(6-4)PP-PL), eiwitten die ver, dan wel nauw verwant zijn aan het zoogdier cryptochroom 1 (CRY1), werden ook onderzocht op hun klokregulerende vermogen. In Hoofdstuk 5 werden transgene muizen gebruikt die in alle cellen van het lichaam het *At*(6-4)PP-PL, dan wel het *Pt*CPD-PL gen tot expressie brengen vanaf de *β-actine* promoter. Met deze muismodellen hebben we laten zien dat de aanwezigheid van *Pt*CPD-PL, in tegenstelling tot At(6-4)PP-

PL, de circadiane klok beïnvloedt door de lengte van de circadiane gedragsperiode te verkorten. Wanneer *Pt*CPD-PL tot overexpressie werd gebracht in gekweekte MDFs, kon het (net als Cc-PHR2) de amplitude van de circadiane oscillatie verminderen, waarschijnlijk als gevolg van de waargenomen interactie tussen de *Pt*CPD-PL en CLOCK-eiwitten. De waarneming dat *Pt*CPD-PL ook CLOCK/BMAL1-aangedreven transcriptie remt, toont aan dat ook dit CPD-fotolyase kan interfereren met de circadiane klok van zoogdieren. Dermale muizenfibroblasten (MDFs) met een deletie van zowel het *Cry1* als het *Cry2* gen (*Cry1*$^{-/-}$/*Cry2*$^{-/-}$) werden gebruikt in een vervolgstudie om te zien of *Pt*CPD-PL het vermogen bezit om te functioneren als een echt cryptochroom. Daartoe werden *Pt*CPD-PL en CRY1 stabiel geïntroduceerd in *Cry1*$^{-/-}$/ *Cry2*$^{-/-}$ MDFs (cellen die van zichzelf geen circadiane ritmiek meer vertonen) en ritmisch tot expressie gebracht vanaf de C*ry1*-promoter. Wanneer *Cry1* of *Pt*CPD-PL aanwezig was, werd het ritme van de Cry-deficiënte cellijn hersteld. Bovendien was *Pt*CPD-PL in staat om de circadiane oscillator in de lever van *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ muizen te repareren, wat liet zien dat *Pt*CPD-PL inderdaad kan functioneren als een cryptochroomeiwit. Deze bevindingen werpen een nieuw licht op het belang van de hoofdstructuur van zoogdier cryptochromen met betrekking tot de functie in de circadiane klok, en als zodanig draagt het bij tot ons begrip van de evolutie van de cryptochroom/ fotolyase-familie.

In Hoofdstuk 6, is een diepgaandere analyse van Cc-PHR2 en *Pt*CPD-PL uitgevoerd. Net als voor *Pt*CPD-PL, werd aangetoond dat Cc-PHR2 in staat is ritmiek te herstellen in de CRY-loze zoogdieroscillator, zowel in gekweekte MDFs als in muizen. Bioluminescentiestudies met *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ fibroblasten, die ritmisch Cc-PHR2 aanmaken vanaf de *Cry1*-promoter, gaven aan dat dit fotolyase de circadiane klok tot leven kan wekken in aritmische MDFs en in de lever van *Cry*-deficiënte muizen. De volgende stap was om te onderzoeken via welk moleculaire mechanisme de klasse II CPD fotolyases interfereren met de circadiane oscillator. Chromatine-immunoprecipitatie-experimenten lieten zien dat Cc-PHR2 en *Pt*CPD-PL niet interfereren met de binding van CLOCK aan E-box elementen in het DNA. Daarnaast liet een co-immunoprecipitatietest zien dat zowel Cc-PHR2 als *Pt*CPD-PL de vorming van CLOCK/BMAL1-heterodimeren niet blokkeert. Daaruit werd geconcludeerd dat fotolyases niet concurreren met BMAL1 om binding aan het CLOCK-eiwit. Deze resultaten suggereren dat de bestudeerde klasse II CPD-fotolyases, Cc-PHR2 en *Pt*CPD-PL, waarschijnlijk actief zijn in de circadiane klok door middel van een mechanisme vergelijkbaar met dat van zoogdiercryptochromen. Deze waarnemingen leiden tot de conclusie dat de algehele structuur van het fotolyase-eiwit een grotere rol speelt dan de aminozuursequentie of domeinsamenstelling, wanneer het erom gaat of er een functionele interactie zal optreden tussen de CLOCK/BMAL1-heterodimeer en het betreffende CFF eiwit.

Concluderend kan gesteld worden dat een voorouderlijk CPD-fotolyasegen in de *Baculoviridae*-familie werd geïntroduceerd via een horizontale genoverdracht vanuit een gastheermot. Deze conclusie is gebaseerd op de bevinding dat de *phr*-genen van deze insecten nauw verwant zijn aan de genhomologen van baculovirussen. Ondanks het feit, dat ChchNPV codeert voor een biochemisch actief fotolyase, heeft dit fotolyase niet het vermogen om de infectiositeit van het virus te herstellen nadat het DNA is beschadigd door UV-licht. Daarnaast werd aangetoond dat klasse II CPD-fotolyases niet alleen UV-schade in DNA repareren, maar ook kunnen ingrijpen op de (zoogdier) circadiane klok. De bevindingen in dit proefschrift hebben geleid tot nieuwe inzichten in de functionele evolutie van CFF-leden in het algemeen, en geven aanwijzingen voor het bestuderen van gastheermodulatie door fotolyase-coderende baculovirussen.

Streszczenie

Rodzinę wirusów *Baculoviridae* tworzą duże DNA-wirusy otoczone błoną lipidową. Bakulowirusy infekują owady, głównie owady z rzędu Lepidoptera. Bakulowirusy są wysoce wirulentne, specyficzne względem gospodarza i bezpieczne dla środowiska. Z tych właśnie powodów bakulowirusy są odpowiednie do zastosowania w walce ze szkodliwymi owadami. W praktyce efektywne użycie bakulowirusów do bioochrony roślin jest uniemożliwione przez wpływ promieniowania słonecznego, promieniowa ultrafioletowego (UV), które dezaktywuje bakulowirusy.

Promieniowanie UV uszkadza DNA, gdyż indukuje powstawanie w DNA dimerów pirymidynowych: cyklobutanowych dimerów pirymidynowych (cyclobutane pyrimidine dimers - CPDs) oraz fotoproduktów (6-4)pirymidynowo-pirymidonowych (pyrimidine-pyrimidone(6-4) photoproducts - (6-4PPs)), które powodują zmiany w strukturze DNA w miejscu uszkodzenia. Aby naprawić CPD lub (6-4)PP prawie wszystkie organizmy (oprócz ssaków łożyskowych, które używają innych mechanizmów) wyposażone są w fotoliazy, CPD fotoliazy i (6-4) fotoliazy. Fotoliazy są specyficzne do rodzaju uszkodzeń w DNA i wykorzystują zaabsorbowaną energię świetlną do funcji katalitycznych (fotoreaktywacja). Ze względu na ich zachowaną strukturę fotoliazy zaliczamy do rodziny białek fotoliaz/kryptochromów (cryptochrome/photolyase family - CPF). Członkowie tej rodziny białek pełnią różnorodne funkcje, bezpośrednio bądź pośrednio związane z odpowiedzią na światło. Fotoliazy są enzymami, które pod wpływem światła widzialnego naprawiają indukowane promieniami UV uszkodzenia w DNA. Kryptochromy natomiast pełnią funkcję fotoreceptorową i/lub są głównym elementem zegara biologicznego.

Fotoliazy CPD na podstawie różnic w sekwencji podzielone są na dwie klasy. Klasa II CPD fotoliaz jest zachowana w specyficznej grupie bakulowirusów, które infekują owady należące do podrodziny Plusiinae w rodzinie sówkowatych (Noctuidae). Wirus poliedrozy jądrowej (nucleoplyhedrovirus, NPV) *Chrysodeixis chalcites* NPV (ChchNPV), zakażający motyle będące poważnym szkodnikiem upraw pomidorów *C. Chalcites,* posiada dwa geny fotoliaz: *Cc-phr-1* i *Cc-phr-2*. Cc-PHR2, w odróżnieniu od Cc-PHR1, posiada aktywność fotoliazy, co wykazano w systemie heterologicznym. Jednak większość bakulowirusów nie posiada genów *phr*. Ponadto zademonstrowano, że Cc-PHR2 kieruje się do jądra komórkowego i ma powinowactwo do DNA. Dodatkowo Cc-PHR2 kolokalizuje się w obrębie miejsca replikacji wirusów w komórce. Badania opisane w tej rozprawie doktorskiej mają pomóc w lepszym zrozumieniu funkcji i ewolucji fotoliaz CPD (głównie tych pochodzących z bakulowirusów).

Rozdział 2 poświęcony jest badaniom filogenetycznym, które mają na celu poznanie ewolucji i funkcji fotoliaz bakulowirusowych, na podstawie analizy ich związku z innymi genami fotoliaz. Aby określić, skąd bakulowirusy uzyskały fotoliazy, częściowe sekwencje cDNA genów fotoliaz wywodzących się z owadów z podrodziny plusiine *C. chalcites*, *T. ni* i *S. exigua* (inny owad z rodziny sówkowatych) zostały zbadane i poddane analizom filogenetycznym. Aby ułatwić interpretację filogenezy fotoliaz, stworzono filogenezę genów poliedryna/granulina (*polyhedrin/granulin*). Na podstawie tych badań stwierdzono, że geny *phr* owadów z rzędu motyli są silnie spokrewnione z ich homologami pochodzącymi z bakulowirusów, podczas gdy bakulowirusowe geny *phr* są bardziej spokrewnione ze sobą niż z fotoliazami pochodzącymi z owadów, będących ich gospodarzami. Uzyskane rezultaty pozwoliły wysnuć wniosek, że gen CPD fotoliazy został przeniesiony horyzontalnie (poziomy transfer genów) od owadów z rzędu Lepidoptera

do przodków bakulowirusów.

Cc-PHR2, w odróżnieniu do Cc-PHR1, posiada zdolości fotoreaktywacyjne i jest w stanie naprawiać uszkodzenia w DNA. Nadal nie jest wiadomo jednak, czy te fotoliazy są w stanie chronić ChchNPV przed promieniowaniem UV. Dlatego w Rozdziale 3 zbadano wrażliwość ciałek krystalicznych (occusion boddies - OBs) ChchNPV na promieniowanie UV. W celu zmierzenia efektu promieniowania UV (z lub bez fotoreaktywacji) na umieralność zaindukowaną przez ChchNPV na larwach *C. chalictes* skonstruowano testy biologiczne. Zaobserwowano, iż umieralność larw pozostała bez zmian, nawet gdy OBs uległy fotoreaktywacji. Wynik ten jest spójny z brakiem fotoliazy w wirionach znajdujących się w ciałkach krystalicznych, co zbadano poprzez analizę proteomiczną (proteomic analysis) wirionów podczas równoległych badań. Prawdopodobnie fotoliazy ulegają więc ekspresji tylko we wczesnej fazie infekcji i tylko wtedy są w stanie naprawiać DNA oraz pełnić pozostałe funkcje.

Fotoliazy są homologami kryptochromów, których funkcją jest regulacja zegara biologicznego ssaków. Kryptochromy działają w zegarze biologicznym jako represory kompleksu CLOCK/BMAL1 dzięki interakcjom z główną domeną CLOCKa i prawdopodobną C-końcową domeną typu coiled-coil BMAL1. Z powodu podobieństwa kryptochromów i bakulowirusowych fotoliaz oraz braku zdoloności fotoreaktywacyjnych Cc-PHR1, zbadano możliwość pełnienia przez fotoliazę CPD funkcji podobnej do funkcji pełnionej przez kryptochromy w zegarze biologicznym. W Rozdziale 4 przedstawione zostały badania dotyczące potencjalnego oddziaływania bakulowirusowych fotoliaz z głównymi elementami zegara biologicznego (CLOCK i BMAL1). Dzięki zastosowaniu technik koimmunoprecypitacji i immunofluorescencji wykazano, że fotoliazy CPD mogą oddziaływać fizycznie z białkiem CLOCK, ale nie z białkiem BMAL1. Następnie przeprowadzono testy z wykorzystaniem lucyferazy w celu sprawdzenia, czy oddziaływanie pomiędzy poszczególnymi białkami przekłada się na odpowiedź fizjologiczną. Tylko Cc-PHR2 powodowała inhibicję transkrypcji prowadzonej przez CLOCK/BMAL1, a zatem działała w sposób podobny do kryptochromu. Wykazano również, że nadekpresja Cc-PHR2 (ale nie Cc-PHR1) zmniejsza amplitudę oscylacji kultur mysich fibroblastów skóry (MDFs), co prawdopodobnie spowodowane jest zdolnością fotoliazy do efektywnego zahamowania transkrypcji prowadzonej przez CLOCK/BMAL1. Jak wiadomo zakażenie bakulowirusem zmienia zachowanie owadów, powodując na przykład zwiększoną ruchliwość czy też wspinanie. Nasze wyniki zachęcają zatem do postawienia hipotezy, że obecność fotoliazy z potencjalną funkcją w zegarze biologicznym mogłaby przynieść bakulowirusom dodatkową korzyść przez pełnienie funcji w indukowaniu zmian w zachowaniu owadów poprzez działanie na ich zegar biologiczny.

Dwie inne fotoliazy, CPD foroliaza klasy II pochodząca z kanguroszczura właściwego (*Potorous tridactylus*) *Pt*CPD-PL i (6-4)PP fotoliaza pochodząca z rzodkiewnika pospolitego (*Arabidopsis thaliana*) *At*(6-4)PP-PL, będące, odpowiednio, spokrewnione daleko i blisko z kryptochromem 1 (CRY1), zostały także zbadane w celu ustalenia ich potencjalnej funkcji w zegarze biologicznym. W Rozdziale 5 wykorzystano transgeniczne myszy z genem *At*(6-4)PP-PL lub *Pt*CPD-PL pod kontrolą promotora β-aktyny, by zademonstrować, że obecność *Pt*CPD-PL, w odróżnieniu do *At*(6-4)PP-PL, przyczynia się do zmiany rytmów okołodobowych, a dokładnie do skrócenia długości cyklu okołodobowego. Nadekspresja *Pt*CPD-PL w MDFs, podobnie jak w przypadku Cc-PHR2, zmniejsza amplitudę okołodobowej oscylacji, prawdopodobnie poprzez zaobserwowaną interakcją *Pt*CPD-PL z białkiem CLOCK. *Pt*CPD-PL jest w stanie również zahamować

transkrypcję prowadzoną przez CLOCK/BMAL1, co potwierdza tezę, że *Pt*CPD-PL pełni rolę regulacyjną w zegarze biologicznym ssaków. MDFs ze znokautowanymi genami *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ zostały następnie użyte, aby zbadać zdolność *Pt*CPD-PL do działania jak prawdziwy kryptochrom. W tym celu *Pt*CPD-PL i Cry1 były stale eksprymowane pod kontrolą promotora Cry1 w arytmicznych *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ MDFs. Obecność Cry1 oraz *Pt*CPD-PL przywróciła okołodobową oscylację w liniii komórkowej nieposiadającej Cry. Ponadto *Pt*CPD-PL była w stanie przywrócić okołodobową oscylację w wątrobie myszy *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ (nieposiadającej zegara biologicznego). To doświadczenie pokazało, że *Pt*CPD-PL może pełnić funkcję kryptochromu. Opisane w tym akapicie wyniki podkreślają kluczową rolę ssaczych kryptochromów w zegarze biologicznym oraz przyczyniają się do lepszego zrozumienia ewolucji rodziny fotoliaz/kryptochromów.

W Rozdziale 6 opisana została dokładniejsza analiza Cc-PHR2 i *Pt*CPD-PL. Cc-PHR2, podobnie jak *Pt*CPD-PL, była w stanie przywrócić rytm okołodobowy w arytmicznym nieposiadającym CRY ssaczym oscylatorze, zarówno w kulturach komórkowych, jak i u myszy. Bioluminescencyjny test z użyciem fibroblastów *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ i stała ekspresja Cc-PHR2 pod kontrolą promotora Cry1 sugeruje, że ta fotoliaza jest w stanie przywrócić okołodobową oscylację w arytmicznych *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ MDFs, jak również w wątrobie myszy nieposiadających CRY. Następnie badaliśmy podstawy mechanizmu molekularnego, który mógłby zostać użyty przez fotoliazy CPD klasy II do regulacji zegara biologicznego. Doświadczenia z zastosowaniem techniki immunoprecypitacji chromatyny pokazały, że obecność Cc-PHR2 i/lub *Pt*CPD-PL nie wywiera negatywnego wpływu na wiązanie białka CLOCK przez chromatynę, na przykład do elementów E-box w DNA. Poza tym eksperyment immunoprecypitacji chromatyny pokazał, iż Cc-PHR2 i/lub *Pt*CPD-PL nie hamują tworzenia heterodimeru CLOCK/BMAL1. Tak więc fotoliazy współzawodniczą z BMAL1 w wiązaniu białka CLOCK. Te wyniki sugerują, że badane fotoliazy CPD klasy II, Cc-PHR2 i *Pt*CPD-PL, prawdopodobnie działają w ssaczym zegarze biologicznym w podobny sposób, używając podobnego mechanizmu molekularnego, jak ssacze kryptochromy. Ta obserwacja prowadzi do konkluzji, że to ogólna struktura białka a nie sekwencja aminokwasów czy też istniejące domeny determinują funkcjonalne oddziaływania, które ma miejsce pomiędzy kompleksem CLOCK/BMAL1 a członkami CPF.

Posumowując, pierwotny gen CPD fotoliazy został najprawdopodobniej wprowadzony do rodziny *Baculoviridae* w wyniku poziomego transferu genów od owadów z rzędu Lepidoptera, gdyż geny *phr* w tym rzędzie owadów są wysoce spokrewnione z ich bakulowirusowymi homologami. Pomimo faktu, że ChchNPV koduje biochemicznie czynną fotoliazę, nie jest ona w stanie przywrócić zdolności zakażania owadów przez ciałka krystaliczne po inaktywacji spowodowanej promieniowaniem UV. Dodatkowo wykazaliśmy, że fotoliazy CPD klasy II nie tylko naprawiają uszkodzone przez promieniowanie UV DNA, ale również ingerują w funkcję (ssaczego) zegara biologicznego. Te wyniki umożliwiają nowe spojrzenie na funkcjonalną ewolucję członków CPF i dają sugestie do ustalenia prawdopodobnych sposobów modulacji zachowania owadów przez bakulowirusy posiadające geny fotoliaz.

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THANK YOU ALL!

Magda

List of publications

Biernat M.A., Ros V.I.D., Vlak J.M., and van Oers M.M. (2011) "*Baculovirus cyclobutane pyrimidine dimer photolyases show a close relationship with lepidopteran host homologues*" Insect Molecular Biology 20: 457-464.

Biernat M.A., Eker A.P.M., van Oers M.M., Vlak J.M., van der Horst G.T.J. and Chaves I. (2012) "*A baculovirus photolyase with DNA repair activity and circadian clock regulatory function*" Journal of Biological Rhythms 27: 3-11.

Chaves I., Nijman R.M., **Biernat M.A.**, Bajek M.I., Brand K., Carvalho da Silva A.M., Saito S., Yagita K., Eker A.P.M., and van der Horst G.T.J. (2011) *"The Potorous CPD photolyase rescues a cryptochrome-deficient mammalian circadian clock*" PLoS One 6: e23447.

Biernat M.A., Caballero P., Vlak J.M., and van Oers M.M. *"Baculovirus photolyases do not reduce the sensitivity of occlusion bodies to ultraviolet (UV) irradiation"* Submitted for publication.

Curriculum Vitae

Magdalena Anna Biernat was born on January 13th, 1983, in Warsaw, Poland. She was studying Biotechnology specializing in Health Care and Animal Production for 4 years at Warsaw Agricultural University, Poland. In 2006 she was awarded with a fellowship to pursue the MSc programme in Medical Biotechnology at Wageningen University (WUR, Wageningen, The Netherlands). She has performed her master thesis project at the Biochemistry Department at Wageningen University. The research aimed at a better understanding of the mechanisms of embryonic root initiation in *Arabidopsis thaliana*. In 2007 she has obtained her Master degree from Wageningen University. In December 2007 she has started the PhD programme in the Laboratory of Virology at Wageningen University in the research group of Prof. Dr Just M. Vlak and Dr Monique M. van Oers, where she has studied the functional and evolutionary aspects of baculoviral CPD photolyases. The research aiming at unraviling the role of CPD photolyases in the circadian clock was performed in the collaboration with the Department of Genetics, Section Chronobiology and Health in the Erasmus Medical Center in Rotterdam, The Netherlands, in the research group of Prof. Dr Gijsbertus T.J. van der Horst and Dr Inês Chaves. Additionally, she has performed the research on the UV sensitivity of the baculoviral occlusion bodies at the Instituto de Agrobiotecnología in CSIC-Gobierno de Navarra in Spain in the research group of Prof. Dr Primitivo Caballero. Her research on the function and evolution of baculoviral and marsupial class II CPD photolyases is described in this thesis.

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (5.6 ECTS)

- Baculovirus CPD photolysases, their function, ecological benefit and hope for skin cancer and NER-deficiency diseases treatment

Writing of project proposal (3 ECTS)

- Functional analysis of baculovirus DNA photolyases

Post-graduate courses (7.5 ECTS)

- Virology course; EMC (2008)
- Biomedical research techniques; MGC Rotterdam (2009)
- Transgenesis, gene targeting and in vivo imaging; MGC Leiden (2009)
- Genome maintenance and cancer; MGC Leiden (2009)
- Safe handling of radioactive materials and sources; PE&RC (2010)

Laboratory training and working visits (4.5 ECTS)

- Function of baculovirus photolyases in the circadian clock; Erasmus Medical Centre (2009-2011)
- UV sensitivity bio-assays; Universidad Pública de Navarra (2011)

Competence strengthening / skills courses (3.1 ECTS)

- PhD Competence assessment; PE&RC (2008)
- Techniques for writing and presenting a scientific paper; PE&RC (2010)
- Career perspectives course; PE&RC (2011)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

- PE&RC Introduction weekend (2008)
- PE&RC Days (2009, 2010)

Discussion groups / local seminars / other scientific meetings (7.6 ECTS)

- Experimental Evolution Discussion Group (EEDG) (2008-2011)
- Work discussion in Laboratory of Virology; Wageningen University (2008-2011)
- Work discussion in Genetics Department; Erasmus MC (2008-2011)

International symposia, workshops and conferences (7.5 ECTS)

- Annual Meeting of the Society for Intervertebrate Pathology (2010)
- Society of Research on Biological Rhythms (2010)
- Gordon Research Conference on Chronobiology (2011)

Lecturing / supervision of practical's / tutorials (9.6 ECTS)

- Molecular Virology; 8 days (2008-2010)
- Cell Biology and Health; 24 days (2010-2011)

Supervision of one MSc student; 3 months (1.5 ECTS)

- Characterisation of DNA photolyase

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