Correspondence

The Drosophila clock protein Timeless is a member of the Arm/HEAT family

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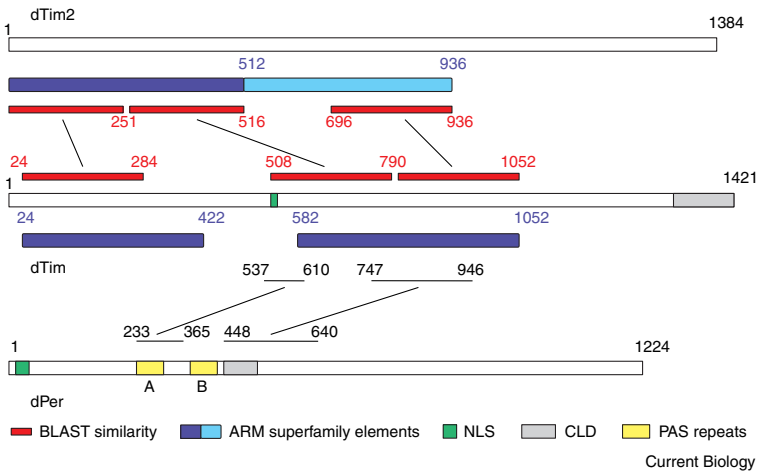

Figure 1.
Representation of putative Arm superfamily domains in *Drosophila* Tim proteins as defined by 3D-PSSM (blue), SAM-T99 and REP. The second Arm domain in dTim2 is shown in light blue because it does not generate a significant score with the algorithms we used (see text), although it appears to contain some Arm repeats. The PSI-BLAST similarities in the primary sequences with residue numbers are shown in red. Nuclear localisation signal (NLS), Cytoplasmic localisation domain (CLD) and PASA and PASB repeats of Per are shown. Regions of interaction between dTim and dPer are shown by linked black bars between the two proteins [11]. Numbering of the residues of dTim is taken from the revised sequence of dTim [23].

In higher eukaryotes, the circadian clock is generated by a system of negative and positive auto-regulatory feedback loops [1]. In *Drosophila,* the negative limb of this mechanism involves the Timeless (Tim) and Period (Per) proteins, which must physically associate in order to be translocated to the nucleus. Once inside, the Per–Tim heterodimer negatively regulates the expression of the *per* and *tim* genes. Per is a founder member of the PAS (Per-Arnt-Sim) protein family [2] and contains two PAS domains that are involved in protein dimerization. The *Drosophila* genome encodes two different *tim* genes, the clock relevant *dtim,* and a second, ancestral gene, *dtim2* or *timeout,* which is the true orthologue of mammalian *Tim* [3,4]. *mTim* does not appear to play a significant role in the murine clock [5]. No protein domains have yet been identified in Tim or Tim2, so in order to gain some insight into the possible biochemical and conformational nature of Tim proteins, we have applied structural predictions that suggest them to be members of the Arm/HEAT family.

A PSI-BLAST reiterative analysis of the ancestral *Drosophila* Tim2 protein revealed similarities between the first 516 residues of dTim2, and two amino-terminal regions of dTim (23%, BLOSUM 80, regions 24–284 and 508–790; Figure 1). Further rounds of PSI-BLAST failed to reveal any significant similarities to other protein classes. Protein structure prediction using PSA (bmerc-www.bu.edu/psa/) suggested that this region in both proteins was entirely α-helical. We therefore used 3D-PSSM [6] to compare further the amino-terminal dTim2 region to the SCOP database [7]. The search returned five highly significant values ($E < 0.05$; note that in 3D-PSSM, $E < 0.5$ is considered significant), representing members of the Armadillo (Arm) repeat superfamily from the fold of alpha–alpha superhelical proteins. Three were from the Arm (yeast and murine importin-α, murine β-catenin) and two from the HEAT (human importin-β and the PR65/A subunit of protein phosphatase 2A) families (Figure 1). Similar searches using hTIM and mTIM also returned significant similarities ($E < 0.06$) with these same Arm/HEAT containing proteins. We also examined with 3D-PSSM the mouse and fly clock proteins, Per, Clock, Bmal1 (and Cycle), CK1ε (and Dbt), and GSK-3 (and Sgg), but no Arm/HEAT domains were detected.

dTim has two amino-terminal isoforms with methionine initiation at +1 and +24, respectively [8], so we analysed the structural motifs common to the amino termini of both variants (Figure 1). This region aligned with human importin-β using 3D-PSSM ($E = 0.08$). Because the similarity with Arm/HEAT repeats was slightly less striking than for dTim2, we analysed dTim by SAM-T99 [9] based on Hidden Markov Modelling and by hydrophobic cluster analysis, HCA [10]. Both of these methods confirmed the existence of an Arm superfamily homologous region in the amino-terminal region (data not shown). The final borders of the Arm region in dTim were defined by 3D-PSSM reiteration to lie between residues 24–422.

To characterise any additional structural elements present in the dTim proteins, we analysed the regions downstream of residues 500. HCA analysis revealed a region consistent with a second Arm superfamily domain located between residues 570–990 in dTim. Examination of this region by 3D-PSSM suggested that the domain borders were slightly larger, apparently extending between residues 582–1052 with homologies to Arm superfamily members (yeast importin-α and human importin-β, $E < 0.5$; murine β-catenin, $E < 0.7$). In dTim2 the presence of additional Arm/HEAT features was not as apparent as for dTim by 3D-PSSM ($E > 1$) although the presence of Arm

superfamily motifs downstream of residue 511 was suggested by REP analysis of residues 512–936 (www.embl–heidelberg.de/~andrade/papers/rep/search.html). Because of the similarity between residues 261–516 of dTim2 and 508–790 of dTim detected by PSI-BLAST analysis, we speculate that the amino-terminal Arm/HEAT domain of dTim2 may be divided in dTim by residues 284–582 which are largely specific to dTim (see Figure 1). This means that the first interaction domain in dTim that was identified by deletion analysis and associates with the first Per PAS repeat [11] (see Figure 1) falls largely, if not entirely, between the two dTim Arm/HEAT regions.

Arm/HEAT motifs are tandem repeats of ~50 residues with highly degenerate sequences [12]. The crystal structures of two Arm family members, importin-α and β -catenin, have defined the ARM repeat as having three helices [13,14], whereas the HEAT repeat, characterized from the structures of importins β1 and β2 and protein phosphatase 2A, consists of only two helices [15–17]. Nevertheless, these two motifs bear a striking resemblance to each other at the conformational level. As dTim is required for the nuclear import of dPer [18] the structural similarity with importins takes on an immediate biological relevance. Furthermore, dPer is retained in the cytoplasm via its cytoplasmic localisation domain (CLD), which associates with dTim residues 747–946 within the second putative Arm domain (Figure 1 and [11]). Consequently a scenario may be envisaged in which the second Arm domain of dTim 'mimics' the plasma membrane component (such as β-catenin), to attract the CLD of dPer.

Finally, within the fly circadian mechanism, Doubletime (casein kinase 1ε) and Shaggy (glycogen synthase kinase-3) are key players in regulating the stabilities of both Per and Tim, respectively [19,20]. These two kinases also carry out similar roles in Wnt signalling, where they regulate the stability of β-Catenin [21,22] which contains Arm repeats. Our finding that Tim may contain domains of Arm/HEAT repeats thus provides the focal point for implicating the Wingless/Wnt signalling module in the circadian mechanism, and suggests that other members of this pathway should be investigated for possible roles in the generation of biological oscillations. Clearly, unequivocal evidence for Arm/HEAT domains within Tim proteins must await a three-dimensional experimental analysis. Nevertheless, our results raise interesting questions concerning the evolutionary origins of this signalling module, and how some components came to be shared among the developmental and circadian pathways.

Acknowledgements
This work was supported by HFSP and BBSRC grants to CPK, a Socrates studentship to NV and a MURST-British Council grant to CPK and RC.

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