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On the molecular mechanism of the circadian clock

The 41,000 M_r clock protein of *Chlorella* was identified as 3-phosphoglycerate kinase

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SUMMARY

A 41,000 M_r polypeptide of *Chlorella* exhibits a circadian rhythm in its synthesis and possesses characteristic features of a putative essential clock protein as was proposed by the coupled translation-membrane model. Purification of this polypeptide and a microsequencing analysis yielded a N-terminal sequence of 35 amino acids that showed no homology to known sequences that were thought to be involved in circadian rhythm such as the *per* gene of *Drosophila* and the *frq* gene of *Neurospora*. However, strong homology was observed to 3-phosphoglycerate kinase (PGK) of different organisms. The highest homology (83%) of this *Chlorella* sequence was found with the PGK of wheat chloroplast.

PGK activity and the 41,000 M_r polypeptide co-purified through differential centrifugation and gel filtration. These data, and comparison with the physical properties of other known PGK molecules, support the conclusion that the 41,000 M_r polypeptide of *Chlorella*, a candidate for a putative essential clock protein, is 3-phosphoglycerate kinase.

Key words: *Chlorella fusca*, circadian rhythm, phosphoglycerate kinase, protein synthesis

INTRODUCTION

Circadian rhythms have been demonstrated in a wide range of behavioral, physiological and biochemical parameters in different organisms from single cells to man (Aschoff, 1989; Bünning, 1973; Edmunds, 1988; Lorenzen et al., 1985; Schweiger et al., 1964). These rhythms are characterized by an endogenous period of around 24 hours (Bünning, 1973) and by the ability of physical or chemical pulses to shift the phase of the oscillation (Karakashian and Schweiger, 1976). Furthermore, these rhythms are temperature compensated (Anderson et al., 1985; Sweeney and Hastings, 1960). The phase-shifts are dependent on the time the pulse is given in the phase of the circadian cycle (Edmunds, 1988; Karakashian and Schweiger, 1976). It is widely accepted that such persisting phase-shifts of free-running rhythms should be interpreted as evidence for effects of the perturbing agent on the clock itself (Jacklet, 1984; Hastings and Schweiger, 1976; Schweiger and Schweiger, 1977). Protein synthesis has been discussed with regard to its possible involvement in the molecular mechanism of circadian rhythms (Edmunds, 1984; Jacklett, 1984; Schweiger and Schweiger, 1977; Takahashi et al., 1993).

One organism in which the circadian rhythm has been studied for many years is the unicellular and uninucleate green alga *Acetabularia* (Schweiger, 1982; Schweiger and Berger, 1979). This cell expresses a well defined circadian rhythm that has been measured by monitoring oxygen evolution (Mergenhagen and Schweiger, 1973; Sweeney and Haxo, 1961), membrane potential differences (Broda and Schweiger, 1981) and intracellular chloroplast migration (Broda et al., 1979; Koop et al., 1978). A further organism with a well documented circadian rhythm is the unicellular green alga *Chlorella*. In this organism, a circadian oscillation of cell division is demonstrated, visible in the time of autospore release (Lorenzen and Albrodt, 1981; Lorenzen et al., 1985). Cultures of *Chlorella* were synchronized by several light-dark cycles and the rhythm of cell division measured under endogenous conditions (constant dark). In the free running rhythm the release of produced autospores is restricted to a small period in the cycle (Malis-Arad and McGowan, 1982; Nishimura et al., 1988). Under conditions of a light-dark cycle the enzyme activities of carboanhydrase (Nara et al., 1989) and nitrite and nitrate reductase (Tischner, 1976) also show an oscillation with one maximum in a 24 hour cycle. However, it is still open whether these rhythms in activity are indeed endogenous and, hence, truly circadian rhythms. In addition it is still unclear whether the changes in activity originate from a varying amount of enzyme or changes in activity itself.

Experiments with *Acetabularia* led to the postulation of the ‘coupled translation-membrane model’ for the molecular mechanism of the clock (Schweiger and Schweiger, 1977). The central part of this model is a two-step mechanism with translation of one or a few putative essential clock proteins (PECPs) on 80 S ribosomes as a first step. In the second step, these proteins were transported to and integrated into a membrane. The integrated proteins alter the properties of the membrane

and cause in this way changes in the environmental conditions. This change in the milieu interieur then results in a suspension of the synthesis of these PECP's. Due to these changes the synthesis of the PECPs is inhibited by a feedback mechanism.

By turnover, the amount of these PECPs integrated into the membranes is decreased. These changes of membrane properties will restore the original environmental conditions and the synthesis of PECPs restarts.

The coupled translation-membrane model is in agreement with a number of essential characteristics of circadian rhythms, i.e. the endogenous character, the temperature compensation and the phase-shifting effect of cycloheximide.

In *Acetabularia*, a high molecular mass polypeptide (P230) has been identified that fulfills the requirements of a PECP within the context of the 'coupled translation-membrane model' (Hartwig et al., 1985, 1986). Recently, we have reported that a 41,000 M_r polypeptide of *Chlorella* also exhibits a circadian rhythm in its rate of synthesis, as measured by [^{35}S]methionine incorporation (Walla et al., 1989). Furthermore, it demonstrates several features characteristic of a PECP. The synthesis of this polypeptide is inhibited by cycloheximide, whereas chloramphenicol has no effect; therefore, translation on 80 S ribosomes and coding in the nuclear genome are supposed. Pulses with cycloheximide result in typical phase-shifts and a series of such pulses lead to a characteristic phase-response curve. A second polypeptide of 42,200 M_r is also synthesized with a rate that demonstrates a circadian rhythm; however, its phase is opposite to that of the 41,000 M_r polypeptide. Interestingly enough, after pulses with cycloheximide no further oscillations in the synthesis of the 42,200 M_r polypeptide were observed (Walla et al., 1989). This 42,200 M_r polypeptide fails therefore to fulfill all of the criteria for a PECP.

The P230 of *Acetabularia* (Hartwig et al., 1985) and the 41,000 M_r polypeptide of *Chlorella* (Walla et al., 1989) were proposed as promising candidates for PECPs. It is still an open question what function these polypeptides have in the cell. In order to investigate the potential role of the 41,000 M_r polypeptide of *Chlorella* in the circadian rhythm, we decided to characterize further the biochemical properties of this protein. We report here the isolation and N-terminal sequence of the 41,000 M_r polypeptide. This partial sequence is highly homologous to sequences of the 3-phosphoglycerate kinase (PGK) of several organisms. In addition, we show that the 41,000 M_r polypeptide possesses PGK activity.

MATERIALS AND METHODS

Cell culture

In all experiments, axenic cultures of *Chlorella fusca* no. 211-8b from the Algal Collections of the University of Göttingen/FRG were used. The algae were grown in a Tris-acetate-phosphate medium (TAP) (Rochaix, 1982) at 22˚C and with illumination at 1200 lux (Philips TL 20W/29 lamps) from one side. Cultures were stirred with a magnetic spinbar at low speed (~100 rpm) without additional aeration. Beginning with 1×10^6 cells/ml, 5 to 8 cycles of 12 hours light and 12 hours dark periods were applied to synchronize the algae. Subsequently, the cultures were transferred to constant light and temperature (Walla et al., 1989).

Treatment of cells

Entrained cultures of *Chlorella* (1 l) were centrifuged at 500 ***g*** for 4 minutes at 20˚C 1 hour or 13 hours after the beginning of constant light, washed in TAP, and resuspended in 50 ml fresh TAP with 1.5 mCi [^{35}S]methionine (Amersham Buchler, Braunschweig/FRG; specific activity 1200 Ci/mmol; 1 Ci = 37 GBq). Following two hours of incubation during light, the cells were pelleted at 500 ***g*** for 2 minutes at 20˚C.

Fractionation of cellular contents

In vivo labelled *Chlorella* cells were resuspended in 4 ml isolation buffer (Walla et al., 1989), frozen in liquid nitrogen and pulverized in a vibrating ball homogenizer (Dismembrator, Braun, Melsungen/FRG) for 3 minutes. Subsequent to thawing, the homogenates were fractionated by centrifugation at 10,000 ***g*** for 15 minutes at 4˚C into a soluble and insoluble protein fraction.

For gel filtration, entrained 5 l cultures were harvested after 3 hours light. The cells were pelleted, washed in TAP and resuspended in TEM (14 mM Tris-HCl, pH 7.7, 1 mM EDTA, 1 mM 2-mercaptoethanol) (John and Syrett, 1967) up to a final volume of 50 ml. The cells were lysed in a Disintegrator-S (IMA, Dreieich/FRG) with 75 ml glass beads of 0.5 mm diameter at 3,500 rpm for 6 minutes on ice. Fractionation was performed by centrifugation at 20,000 ***g*** at 4˚C for 45 minutes.

Gel electrophoresis and electroblotting

One-dimensional gel electrophoresis was performed with 10% polyacrylamide gels essentially as described by Laemmli (1970). Staining and fluorography were done as previously reported (Walla et al., 1989). Two-dimensional gel electrophoresis was done as described by Duncan and Hershey (1984). The first dimension was performed in glass tubes with 2.5 mm inner diameter. The used ampholytes (Pharmacia-LKB, Freiburg/FRG) had a pH range of 3.5-10 and 10% polyacrylamide gels were used for the second dimension. Electroblotting of proteins on a siliconized-glass-fiber membrane and Coomassie Blue staining were performed as described by Eckerskorn et al. (1988a).

Gel filtration

Purification of the 41,000 M_r polypeptide was performed by gel filtration of soluble *Chlorella* proteins on Sephadex G150. A column of 16 mm inner diameter and 90 cm length was loaded with 5-10 ml supernatant of lysed cells in TEM subsequent to 45 minutes centrifugation at 20,000 ***g*** at 4˚C. Elution of the separated proteins was carried out with TEM with a flow rate of 20 ml/h. Fractions of 10 ml volume were collected.

Microsequence analysis

After gel filtration, the 41,000 M_r polypeptide was concentrated by the addition of $(NH_4)_2SO_4$ to 70% saturation and was further separated from other polypeptides by two-dimensional gel electrophoresis and blotted onto a glass-fiber membrane. After Coomassie Blue staining, the corresponding spot was cut out and a microsequence analysis was performed by F. Lottspeich and C. Eckerskorn (Max-Planck-Institut für Biochemie, Martinsried/FRG) according to standard protocols (Eckerskorn et al., 1988a,b).

Determination of 3-phosphoglycerate kinase activity

PGK activity was assayed spectrophotometrically by monitoring the decrease of adsorption at 365 nm due to the decline of the concentration of NADH. The assay contained 2.5 ml 0.1 M triethanolamine (pH 7.6), 0.2 ml 32 mM ATP, 0.05 ml 14 mM NADH, 0.1 ml 27 mM EDTA, 0.05 ml 0.1 M $MgSO_4$ and 0.02 ml 10 mg/ml glycerinaldehyde-3-phosphate-dehydrogenase (specific activity 100 units/mg; Boehringer/FRG) to which 0.05 ml of the soluble, unlabelled protein fraction of *Chlorella* was added. After equilibration to 25˚C, the reaction was started by the addition of 0.2 ml 93 mM glycerate-3-phosphate and the A_{365} was continously monitored for 5 minutes (Bücher, 1955).

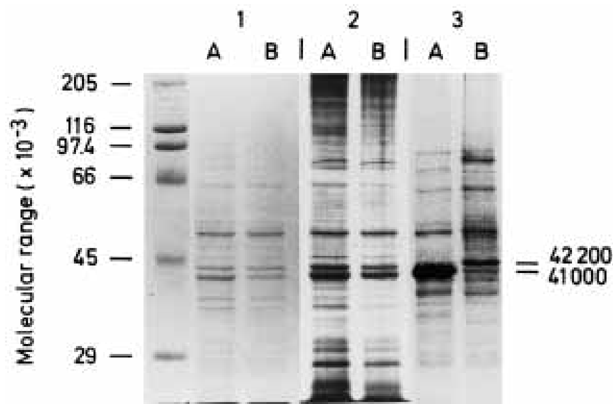

Fig. 1. PAGE of the supernatant fraction of *Chlorella* subsequent to Coomassie Blue staining (1), silver staining (2), and fluorography (3). The cells were labelled with [^{35}S]methionine for 2 hours prior to harvesting 3 hours (A) or 15 hours (B) after the beginning of constant light. The positions of the 41,000 M_r and the 42,200 M_r polypeptides are indicated.

RESULTS

The 41,000 M_r polypeptide of *Chlorella* exhibits a circadian rhythm in its rate of synthesis measured by in vivo labelling of entrained cultures with [^{35}S]methionine (Walla et al., 1989). There is maximal synthesis at 3 hours and minimal synthesis at 15 hours after the beginning of constant light (Fig. 1, lanes 3AB). A second polypeptide of 42,200 M_r showed circadian oscillations in its rate of synthesis in opposite phase to the 41,000 M_r polypeptide. However, the rhythms of both polypeptides were detectable only in the rate of methionine incorporation but not in the amount of polypeptides present at different circadian times (Fig. 1, lanes 1 and 2AB). In all experiments the same amount of radioactivity was applied to each lane.

Two-dimensional gel electrophoresis was performed with *Chlorella* proteins labelled in vivo 3 hours or 15 hours after the beginning of constant light. Identification of the 41,000 M_r and 42,200 M_r polypeptides was performed by comparison of fluorographs of these gels (Fig. 2). Fluctuations in the rate of methionine incorporation in two spots of the expected M_r were clearly detectable (Fig. 2). Comparison of the fluorographs with Coomassie Blue-stained gels point out that both proteins seemed to be present in equal amounts at 3 hours as well as at 15 hours (Fig. 2, inset).

The 41,000 M_r polypeptide was purified by gel filtration of the soluble proteins of *Chlorella* on a Sephadex G150 column (Fig. 3). Further identification of the polypeptide in Coomassie Blue-stained gels loaded with fractions of the eluate was done as already mentioned in the identification of this polypeptide in two-dimensional gel electrophoresis. Therefore, gel filtration of proteins labelled in vivo at 3 hours and at 15 hours after light on were carried out and fractions of the eluate compared in fluorographs after gel electrophoresis. The proteins of the fractions containing the main part of the 41,000 M_r polypeptide (Fig. 3, fractions 31 to 33) were concentrated by $(NH_4)_2SO_4$ precipitation and further separated by two-dimensional gel electrophoresis. After this step, the 41,000 M_r polypeptide was visualized as a single major spot (Fig. 4), with two minor isoelectric variants. Both minor species showed the same oscillation in the rate of methionine incorporation as the main spot. These proteins were electroblotted from two-dimensional gels to glass fiber membranes, which were subsequently stained with Coomassie Blue. The main spot of the 41,000 M_r polypeptide was cut out of these membranes. A microsequence analysis was performed with 20 pooled spots. A sequence of 35 amino acids was identified (Fig. 5). Comparison of this protein sequence with protein and translated DNA sequences of different data banks (EMBL, Heidelberg; Microgenie, Beckman, München/FRG) revealed that the *Chlorella* sequence shares strong homology to the 3-phosphoglycerate kinase (PGK) of different organisms (Fig. 6). The highest extent of homology was to the sequences of PGK of wheat chloroplast (83% of the amino acid sequence) and wheat cytosol (72%) (Longstaff et al., 1989). Homology of the *Chlorella* sequence to PGK of different organisms was restricted to the N-terminal part of the PGK sequences and no further significant homology to the rest of these or other sequences was detectable. In particular, significant homology of the 41,000 M_r N-terminal sequence to other sequences thought to be involved in circadian rhythms, such as the *per* locus of *Drosophila* (Jackson et al., 1986), the *per* homologues of *Acetabularia* (Li-Weber et al., 1987) and mouse (Shin et al.,

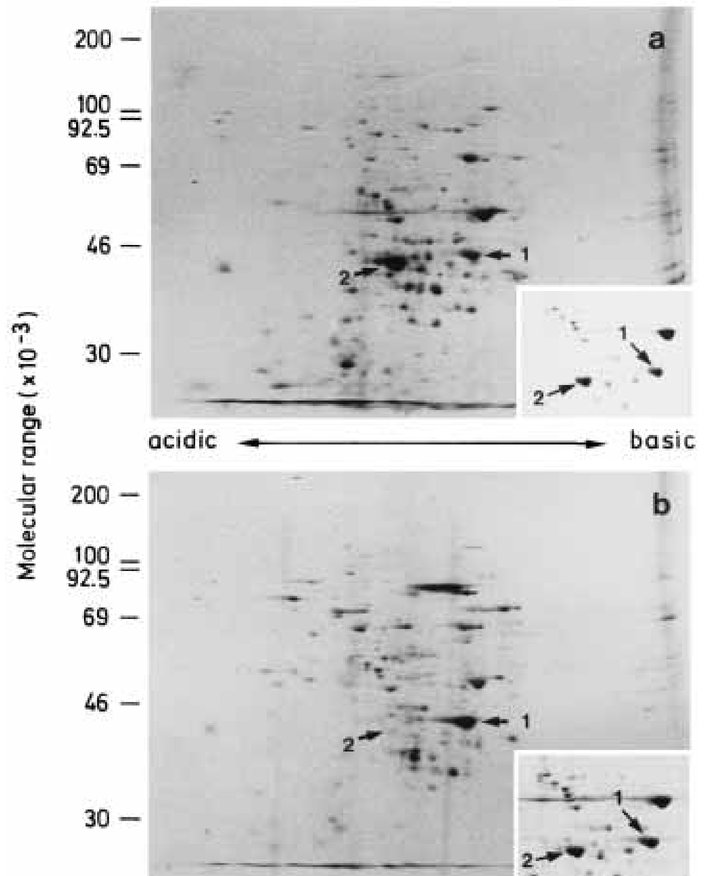

Fig. 2. Fluorographs of *Chlorella* proteins of the supernatant fraction after two-dimensional gel electrophoresis. The cells were labelled with [^{35}S]methionine 3 hours (a) and 15 hours (b) after the beginning of constant light. The 41,000 M_r (2) and 42,200 M_r polypeptide (1) are indicated. The inserts in the panels show the central part of each gel after Coomassie staining.

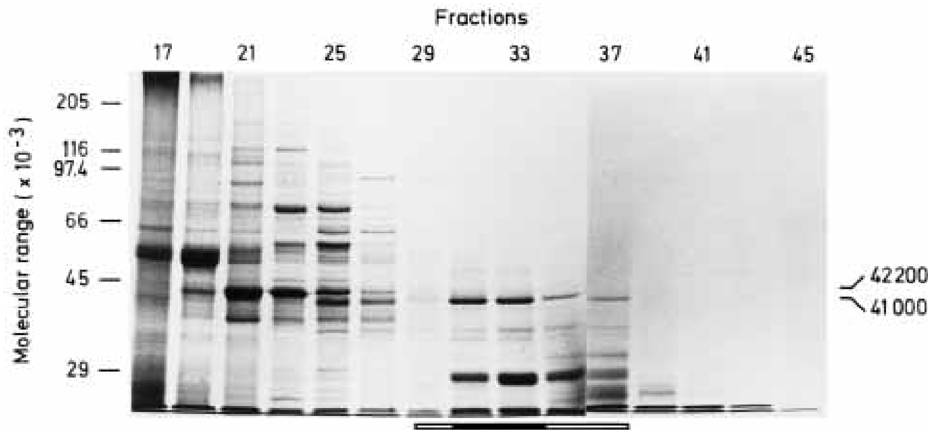

Fig. 3. PAGE of *Chlorella* proteins of the supernatant fraction after gel filtration on Sephadex G150. Each lane represents an aliquot of protein from each fraction of the eluate. The bar at the bottom indicates fractions containing the 41,000 M_r polypeptide (main part, black). The positions of the 41,000 M_r and 42,200 M_r polypeptide are marked.

1985), the *frq* locus of *Neurospora* (Feldman et al., 1979; Hamblen-Coyle et al., 1989; Konopka and Benzer, 1971) and the isocitrate lyase of different organisms (Beeching and Northcote, 1987; Rieul et al., 1988), was not found.

The distribution of PGK activity between the soluble and insoluble fractions of *Chlorella* was determined. The main part of the activity (80%) was located in the supernatant fraction after centrifugation for 15 minutes at 10,000 ***g*** at 4˚C. After an additional washing step at 4˚C of the pellet fraction, only 2% of the original PGK activity remains in this pellet.

The following experiments were designed to answer the question whether the occurrence of the 41,000 M_r polypeptide and of the enzymatic activity of the PGK were correlated after gel filtration. Therefore, PGK activity was measured in each fraction of the eluate of a G150 column loaded with soluble proteins of *Chlorella*. Enzyme activity was only found in the fractions that contain the 41,000 M_r polypeptide (Fig. 7). Furthermore, these pooled fractions contained approximately 85% of the enzyme activity originally loaded onto the column.

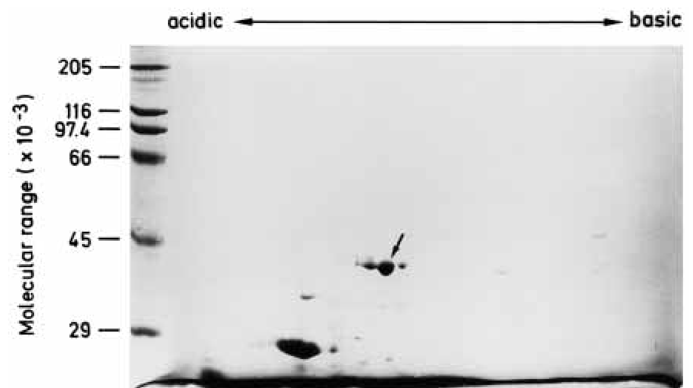

Fig. 4. Coomassie Blue-stained proteins after two-dimensional gel electrophoresis of pooled fractions numbers 31 to 33 after the gel filtration step shown in Fig. 3. The arrow indicates the 41,000 M_r polypeptide.

DISCUSSION

According to the coupled translation-membrane model, the regulation of the circadian clock occurs at the level of translation. Recently published experiments with the dinoflagellate *Gonyaulax* demonstrate that translation is involved in the clock function (Milos et al., 1990). Furthermore, the coupled translation-membrane model postulates the existence of putative essential clock proteins (Schweiger and Schweiger, 1977). Two polypeptides have been identified that are potential candidates for PECPs within the context of the coupled translation-membrane model. One is the protein P230 of *Acetabularia* (Hartwig et al., 1985, 1986) and the other the 41,000 M_r polypeptide of *Chlorella* (Walla et al., 1989). Both exhibit a circadian oscillation in the rate of synthesis that is phase-shifted by cycloheximide pulses independent of the time in the circadian cycle when a pulse is given (Hartwig et al., 1985; Walla et al., 1989). In the case of P230, it was the first time that the cellular level of a protein was shown to be regulated by a circadian clock. Recently, it was published that the synthesis of the luciferin-binding protein (LBP) of *Gonyaulax* also shows a circadian rhythm (Morse et al., 1989, 1990). It was further shown that the LBP mRNA is present in *Gonyaulax* in similar amounts at all times during the day (Lee et al., 1993).

Results obtained with *Chlamydomonas*, in our laboratory, demonstrate also the existence of a PECP in this alga. A protein with a molecular mass of about 64,000 is synthesized with a period length of around 29 hours under endogenous conditions (Wiedemann et al., 1992). Recently, we published the finding that three proteins of *Euglena gracilis* are also synthesized in a circadian rhythm under constant conditions. These proteins with molecular masses of about 16,400, 23,500 and 60,300 are located in the sedimentable cell membrane fraction (Künne et al., 1992a). Furthermore, it was shown that the synthesis of these putative essential clock proteins is temperature compensated (Künne et al., 1992b).

The molecular mechanism of the circadian clock is still unknown and the biological function of the identified PECPs is still unclear. Therefore, we decided to get more information about the biochemical behavior of the 41,000 M_r polypeptide of *Chlorella*. The rhythm of the 41,000 M_r polypeptide of this organism is only detectable in the rate of methionine incorporation. Readily detectable oscillations in the relative amount of protein visible by Coomassie Blue staining did not occur. Comigration of this 41,000 M_r polypeptide with polypeptides of similar M_r that might hide the existing rhythm of the amount of protein is highly unlikely in light of the results of the two-

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      1                                         10
NH2-Ala Lys Lys Ser Val Gly Asp Leu Thr Lys Ala Asp Leu Glu Gly Lys Arg Val

          20                                        30                36
     Phe Val Arg Ala Asp Leu Asn Val Pro Leu Asp Lys Glu Gln Lys xxx Thr Asp
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Fig. 5. N-terminal amino acid sequence of the 41,000 M_r polypeptide determined by a microsequence analysis. The identity of the amino acid in position 34 is unknown.

dimensional gel electrophoresis. The 41,000 M_r spot in a two-dimensional gel shows the same circadian oscillations in its synthesis as it does after analysis by one-dimensional PAGE. These fluctuations were also not detectable after Coomassie Blue staining. In *Chlorella*, a second protein (M_r 42,200) is synthesized in a circadian manner. Nevertheless, it fails to fulfill the further criteria for a PECP according to the coupled translation-membrane model (Walla et al., 1989). However, it is remarkable that the rhythm of the 42,200 M_r polypeptide also is detectable only by methionine incorporation, i.e. in rate of synthesis, but not in the amount of polypeptide itself.

Purification of the 41,000 M_r polypeptide was obtained by gel filtration on a Sephadex G150 column. Further separation from remaining polypeptides was achieved by two-dimensional gel electrophoresis. After this separation step, one major spot of the 41,000 M_r polypeptide was visible. Minor spots adjacent to the 41,000 M_r polypeptide exhibited the same oscillation of methionine incorporation as the main spot and were therefore presumed to be isoelectric variants of the 41,000 M_r polypeptide.

The sequence of 35 amino acids obtained from the main spot of the 41,000 M_r polypeptide of *Chlorella* was compared with the known clock genes *per* of *Drosophila* (Jackson et al., 1986) and *frq* of *Neurospora* (McClung et al., 1989). There was no significant homology to the *Chlorella* sequence although the *per* and *frq* sequences share a sequence element of 48 amino acids (McClung et al., 1989). In addition, the *Chlorella* sequence showed no homology to the *per*-homologous sequences of mouse (Shin et al., 1985) and *Acetabularia* (Li-Weber et al., 1987). However, this was not surprising in consideration of the fact that homology of the *Acetabularia* and mouse sequences is only due to a repetitive sequence element of the *per* locus coding for the amino acids glycine and threonine. This repetitive element is not present in the *Chlorella* sequence of the 41,000 M_r polypeptide.

The sequence of the 41,000 M_r polypeptide of *Chlorella* was used to search gene and protein data banks. One protein, the 3-phosphoglycerate kinase (PGK) of different organisms, was found to share strong homology with the N-terminal part of this sequence. The highest correspondence was found with PGK of wheat chloroplast (Longstaff et al., 1989). The 89% identity of the *Chlorella* sequence with PGK of wheat chloroplasts indicates that this sequence belongs to PGK of *Chlorella* chloroplasts.

The homology of the 41,000 M_r sequence with PGK is highly significant because the sequence Kx Vxx Rx Dx NVP, which represents a sequence motif completely invariant in all known PGK's from *E. coli* to man is also present in the *Chlorella* sequence. This gives a probability of at least less than 10^{-9} that this homology is a chance event. Thus, the 41,000 M_r chloroplast protein is most likely PGK. The search

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                          10        20        30

Wheat chl.    MAKKSVGDLTAADLEGKRVLVRADLNVPLD==DNQNITD
Wheat cyt.   MATKRSVGTLGEADLKGKKVFVRADLNVPLD==DAQKITD
E. coli          MSVIKMTDLDLAGKRVFIRADLNVPVK===DGVVTS
Zymomonas       MAFRTLDDIGDVKGKRVLVREDLNVPMD===GDRVTD
Aspergillus   MSLTSKLSITDVDLKDKRVLIRVDFNVPLDKNDNTTITN
Trichoderma   MSLSNKLSITDVDLKGKRVLIRVDFNVPLD==ENKKITN
Thermus             MRTLLDLDPKGKRVLVRVDYNVPVQ===DGKVQD
Yeast          SLSSKLSVQDLDLKDKRVFIRVDFNVPLD===GKKITS
Tryp. b        SLKERKSINECDLKGKKVLIRVDFNVPLD===DGKITN
Tryp. c        TLNEKKSINECDLKGKKVLIRVDFNVPVK===NGKITN
Human          SLSNKLTLDKLDVKGKRVVMRVDFNVPMK===NNQITN
Mouse          SLSNKLTLDKLDVKGKRVVMRVDFNVPMK===NNQITN
Horse          SLSNKLTLDKLNVKGKRVVMRVDFNVPMK===NNQITN

Consensus      xxxxxxxαxxxxdαxgKrVααRxDαNVPαx//xxxxαtx

Chlorella      AKKSVGDLTKADLEGKRVFVRADLNVPLD==KEQKXTD
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Fig. 6. Comparison of the N-terminal amino acid sequences of PGKs of different organisms with the *Chlorella* sequence. Gaps (=) are included to align wheat chloroplast and wheat cytosol PGK (Longstaff et al., 1989) with the PGKs of yeast (Watson et al., 1982), *Trypanosoma brucei* b and c (Osinga et al., 1985), *Zymomonas mobilis* (Conway and Ingram, 1988), *Aspergillus nidulans* (Clements and Roberts, 1986), *Trichoderma reesei* (Vanhanen et al., 1989), *Thermus thermophilus* (Bowen et al., 1988), *Escherichia coli* (Alefounder and Perham, 1989), human (Michelson et al., 1983), horse (Banks et al., 1979) and mouse (Mori et al., 1986). Identical residues are indicated (hatched). The amino acid residue numbers refer to the wheat chloroplast PGK. The symbols employed in the consensus sequence are: α, hydrophobic amino acid residues; capital letters, identity; small letters, conservative exchanges or > 90% identity; x, any amino acid residue; /, positions where gaps may be introduced to maximize alignment.

failed to identify any further genes or proteins other than PGK with significant homology to the *Chlorella* sequence.

However, there was significant homology (up to 83%) to known PGK sequences of a series of different organisms ranging from yeast to man (Fig. 6). Remarkably, the conserved sequence part is from amino acid 14 to 29, while the remaining residues show more diversity. In the total sequence of listed PGKs (Fig. 6), there are several such evolutionarily conserved sequence elements, which form the inner loops in the substrate-binding cleft (Mori et al., 1986).

An enzyme assay for PGK showed that the main part of enzyme activity was located in the supernatant fraction of lysed *Chlorella* cells. There was a good correlation between the distribution of PGK activity and relative amount of 41,000 M_r polypeptide in the fractions obtained by gel filtration (Fig. 7). The highest PGK-activity was found in the fractions used for the isolation of the 41,000 M_r polypeptide (Fig. 4). The following aspects support the premise that the 41,000 M_r polypeptide and the PGK of *Chlorella* are identical. First, known PGKs are in the same molecular mass range of 38,000

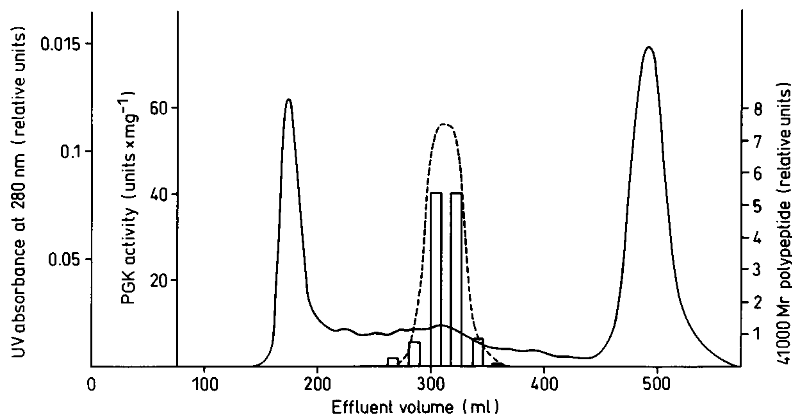

Fig. 7. Distribution of the 41,000 M_r polypeptide (- - -), the PGK activity (bars) and the UV absorbance (—) after separation of the supernatant fraction of *Chlorella* on a Sephadex G150 column.

to 47,000 (Bowen et al., 1988; Conway and Ingram, 1988; Huskins et al., 1982; Köpke-Secundo et al., 1990; Lee, 1982; McMorrow and Bradbeer, 1990; Persson and Olde, 1988; Ray and Rao, 1988). Second, after two-dimensional separation of *Chlorella* proteins from single fractions with high PGK activity subsequent to a gel filtration step, only one major spot appears and is in the molecular mass range of around 41,000. Third, the pooled fractions containing the 41,000 M_r polypeptide also contain about 85% of the PGK enzyme activity originally loaded to the column. Therefore, we conclude that the enzymatic activity of the PGK is a feature of the 41,000 M_r polypeptide. The assumption that the lateral spots of the 41,000 M_r polypeptide in two-dimensional gel electrophoresis are isoforms of the same protein is supported by the fact that there are three isoenzymes of PGK known in *Trypanosoma* (Osinga et al., 1985; Le Blancq et al., 1988) and two in wheat (Longstaff et al., 1989).

The PGK of *Chlorella* fulfills the requirements of a PECP in the context of the coupled translation-membrane model. From the point of view of this model it is noteworthy that the 41,000 M_r polypeptide of *Chlorella* appears to be a chloroplast protein as is deduced from the 89% identity of its partial sequence with wheat chloroplast PGK.

This fact raises the question of whether the PGK performs a clock function as an essential part of the clock mechanism (perhaps in addition to its enzymatic function) or whether the PGK is only under clock control (hand of the clock) in its translational behavior. Further experiments are required to clarify this point. In this regard, interesting data have been presented by Jindal and Vishwanatha (1990a,b). These authors have shown that the PGK of human placenta (M_r 41,000) plays the role of a primer recognition protein (PRP) in a complex with a second protein of M_r 36,000. This 36,000 M_r subunit of the PRP complex is as yet unidentified. The PRPs are cofactors of DNA polymerase α and thereby may have an important role in lagging-strand DNA replication. Initiation of transcription by DNA polymerase α was highly efficient only in the presence of the PRP complex. The purified PRP complex alone exhibited no DNA polymerase activity and completely restored the activity of DNA polymerase α on templates such as heat-denaturated DNA. Thus, the PGK is a potential candidate for a regulatory element in DNA replication. With regard to the circadian clock, it is plausible that the PGK may be a direct link between the clock and the control mechanisms of the cell cycle. Such a link is necessary to explain the fact that, in a series of organisms, the circadian clock and the cell cycle seem to be strictly coupled, as can be concluded from the circadian behavior of cell division (Lorenzen et al., 1985; Edmunds, 1988).

Our data suggest that it should be feasible to screen *Chlorella* cDNA and genomic libraries with oligonucleotides based on the *Chlorella* PGK protein sequence presented here or PGK gene probes derived from other organisms in order to isolate full-length clones of the PGK gene of *Chlorella*. We hope information derived from such clones, such as the physical organization and sequence of transcriptional control elements, will provide clues as to the mechanism of the circadian clock. Additionally, it is at least theoretically feasible that, with the appropriate molecular genetic manipulations, one might specifically modify or delete chromosomal copies of the PGK and thereby directly investigate its purported role in the circadian clock mechanism.

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