Relation between Glycoprotein and EA4 – Time Mechanism in *Secamia creatica*

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Abstract: ATPase (EA4) seems to measure time- interval as a diapause – duration timer in the seasonal cycle of the Sesamia cretica. A peptide named peptidyl – inhibitory needle (PIN) seems to regulate the time measurement of EA4. We characterize the EA4 in the first step to analyse its interaction with PIN. Matrix – assisted laser desorption/ ionization – time of flight- mass spectrometry shows EA4 of an equimolar complex with PIN. The binding affinity of EA4 for PIN is about 460nM, measured by surface plasmon resonance. Western blot analysis of EA4 with a variety of biotynylated lectins suggest that EA4 is a glycoprotein containing N- linked oligosaccharide. By enzymatic cleavage of the glycosyl chain the carbohydrate is revealed to be essential for the regulation of EA4- time measurement through the interaction with PIN. PIN holds the timer by binding to EA4, and the dissociation of the complex could constitute the cue for the time measurement. [Nature and Science. 2010;8(5):131-138]. (ISSN: 1545-0740).

Key words: Timer protein, Glycoprotein, Time – EA4, ATPase

1. Introduction

Biological systems to measure and mark elapsed time may be involved in the accurate timing of developmental events in cells (/). In principle, the systems could measure time in two very different ways. One possibility is that each cellular event is dependent on the previous events, and all events are linked together in a fixed order. Alternatively, cells may have an independent internal clock, together with devices to ensure that key reactions occur at certain times, like an alarm clock. Many important contributions have been made to identify such time-measuring mechanisms. In a self-sustaining clock that regulates daily rhythm, some key insights have led to the identification of putative clock components. Among the best candidates at present is the period protein (PER). PER is required for the proper manifestation of circadian rhythms (2. J). But we have comparatively few clues as to how cells are able to measure elapsed time in a long-term interval timer type of a biological clock. Recently, an ATPase called EA4 was found to have a possible capability of measuring a time interval as a diapause-duration timer in the seasonal cycle of the sesamia cretica (4.5).

The EA4 of sesamia cretica diapauses eggs exhibits a one- time transitory burst activation during the chilling of eggs to terminate diapauses. It is noteworthy that the activation is also observed in vitro. The sudden elevation of EA4 activity hi vitro was equivalent to that observed in vivo and was coincident with the chilling period that is known to be indispensable for diapauses termination. The in vivo and in vitro combination experiment demonstrated that the in vitro activation of EA4 occurred at the same rate as in viva activation (4, 5). EA4 is likely to possess some sort of inherent time-measuring activity.

The possible timer function of EA4 may comprise a built-in mechanism in the EA4 protein structure, and it may undergo a series of conformational changes with time (4). A peptide named PIN (Peptidyl Inhibitory Needle) may inhibit the conformational change of EA4 (6, 7). When EA4 was mixed with PIN, not only was the activation of the enzyme inhibited but also the clock-run of EA4 delayed to the period equivalent to that of PIN inclusion. It is conceivable that the interaction between EA4 and PIN is involved in the regulatory mechanism of the timer. Although these observations reinforce the validity of EA4 as an interval timer-type biological clock, the mechanism by which EA4 measures the time-interval and the question of how EA4 activation is related to the resumption of embryogenesis are still unresolved. In this context, it is crucial that the time-interval activation of EA4 be accomplished after Sephadex G-25 filtration in the EA4 purification (4, 5). Certain considerations about the Sephadex-results form the basis for the design of the following experiment.

EA4 is eluted later than the void volume fraction through the Sephadex G-25 column, even though its

Mr is about 20kDa (4). Thus, the Sephadex column works not as a gel-filtration but as an affinity chromatography.

This suggests a significant proportion of the EA4-mass may be composed of carbohydrate structures, which contribute to the delayed elution from the Sephadex column. This also suggests the EA4 carbohydrate moiety is involved in the mechanism of time measurement. Therefore, the glycoprotein characterization of EA4 is of importance in the research to address the regulatory EA4-timer mechanism. The present experiment is carried out in the first attempt in that direction. Our principal conclusions are that the carbohydrate is essential for the regulation of EA4-time measurement through the interaction with PIN and that the EA4-PIN into reaction may play a central role in the EA4 timer.

2 - Materials and Methods

Preparation of EA4 and PIN - C108(old) Sesamia cretica diapuase eggs were used. Eggs laid within 3h were collected at 25°C to obtain synchronous egg batches. Two days (50h) after oviposition, the eggs were washed with cold acetone and then EA4 was prepared. The preparation procedures are described in details in a previous paper (5), and included acetone powder from the eggs, heat treatment at 85°C, precipitation from 80% saturated ammonium sulfate and gel filtration through a Sephadex G-25 column with EA4 being eluted later than the void volume fraction. The collected EA4 fractions were removed from contaminated PIN by Centricon-10™ centrification (Amicon, Lexington, USA). PIN removal was accomplished by repeated dilutions and filtrations basically by the method of Kai et al. (5), the exception being that the final filtration Was achieved with HEPES buffer (25 mM HEPES, 12.5 mM Trizma Base, pH 7.4) containing 50 mM NaCI, 20 mM KC1, 1.0 mM EDTA, and 100 µ g/ml salmon testes DNA. Unless otherwise noted, all preparation procedures were conducted in a cold room (4°C) or in an ice-water bath.

The Sephadex G-25 filtration is the critical step in the purification of EA4 (4, 5). Therefore, the first part of EA4 preparation was carried to the step of the ammonium sulfate precipitation in one day. The last part of EA4 preparation from the Sephadex filtration was completed another day when enzyme activities were determined.

In the Sephadex G-25 gel filtration, PIN was contained mainly in the void volume fractions, and only in low concentration in fractions after the void volume. As suggested previously, EA4 was retained while PIN was filtered and recovered in the filtrate through Centricon- 10™ (Amicon, USA) centrification (7). PIN was obtained from the filtrate and concentrated by repeated centrification according to the method of Kai et al.(5, 7).

Incubation of EA4 and AT Pase assay - ATPase assay of the EA4 preparation was run at 25°C for 30 min or 3h in sterilized silicon-coated test tubes (5); the reaction HEPES buffer contained 50 mM NaCI, 20 mM KC1, 1.0 mM EDTA, and 100 µ g/ml salmon testes DNA. The time of EA4 activity-appearance was expressed as the time elapsed after the Sephadex G-25 step of the EA4 preparation as done previously (5).

Purification of EA4 for lectln-binding - The EA4 was further purified for lectin binding. First, the EA4 preparation as described above was separated and desalted utilizing disposable cartridges packed with silica bonded to C_{18} hydrocarbon (Sep-Pak C_{18} cartridges, Welters Associates). The cartridge was developed sequentially with Milli Q (Millipore) water containing 0.1% trifluoroacetic acid (TFA) (5 ml), 20% acetonitrile in 0.1% TFA (2 ml), 50% acetonitrile in 0.1% TFA (4 ml) and acetonitrile (6 ml). The 50% acetonitrile fraction was lyophilized and then dissolved in 100µ Milli Q water containing 0.05% TFA to apply to a 4.6mm x 25cm (5 µ m) YMC-Pack PROTEIN-RP (YMC, Japan) reverse-phase column in 0.05% TFA in Milli Q water (solvent A). The column was eluted with a stepwise gradient generated from solvent A and B (0.05% TFA in 100% acetonitile) at a flow rate of 1 ml/min, using the following time course: solvent A (5 min), 0 à 30% B (5 min), 30% B (5 min) and 30 à 45% B (15 min). The absorbance of the column eluate was monitored at 215 nm. The absorbance peak fraction with a retention time of 23 min was lyophilized and then dissolved in 100µ 25% acetonitrile in 0.05% TFA to reapply to YMC-Pack PROTE1N-RP. The second column was eluted at a flow rate of 0.8 ml/ml, using a gradient of 25% B (5 min) and 25 à 35% B (40 min). The absorbance peak of 33 min was collected and used for the following lectin blot analysis. All HPLC procedures were done at room temperature.

Electrophoretic transfer of proteins to PVDF sheets and lectin-binding for glycoprotein detection - SDS-PAGE was performed in Imm-thick slabs with a RAPIDAS Mini-Slab Electrophoresis Cell (ATTO, Japan) by the procedure of Laemmli (5). Briefly, the separating gels containing 12.5% (w/v) acrylamide, 0.5% NN"- methylenebisacrylamide, 0.1% SDS and 375mM-Tris/HCl, pH 8.8; the stacking gels contained 4.5% (w/v) acrylamide, 0.18% NN" methylenebisacrylamide, 0.1% SDS and 125mM-Tris/HCl, pH6.8. The running buffer was 25mM-Tris/19.2mM-glycine/0.1% SDS, pH8.3. After the run, the proteins were either transferred to a

polyvinylidene difluoride membrane (see below) or fixed and stained with silver (Silver Stain Kit Wako, Wako, Japan).

For the trophoelecretic transfer of proteins to polyvinylidene difluoride membrane (Immobilon PVDF Transfer Membrane, Millipore, USA) after completion of the run, the gels and identically sized membranes were placed in a Horiz-Blot Electrophoresis Apparatus (ATTO, Japan) containing electrode buffer (20% methanol/100mM-Tris/192mM-glycine, pH8.3). Transfer was performed at 120 mA for 100 min. After transfer, the membranes were cut longitudinally at 1 cm intervals. One of the sample strips and the strip containing the molecluar-weight makers were then stained in colloidal gold (LECTIN SENSOR Honen, Honen, Japan). In every experiment, the acrylamide gel was also stained with silver (Silver Stain Kit Wako) to assess the efficiency of transfer.

The protein on PVDF strips was detected with lectins. First, the strips were soaked in blocking buffer (10mM Tris- HCI, 0.053% Tween 20, 1% NaCI, pH 7.4) four times for every 15 min and then incubated with biotynylated lections (LECTIN SENSOR Honen) for 80 min (only for ConA, 90 min). After washing four times with blocking buffer I, the bound biotinyl conjugates were introduced to complexes of avidin-biotinyl-peroxidase (ABC) by incubation with horseradish peroxidase-avidin solution (HRP-avidin, Honen). The treated sheet was washed four times with blocking buffer I, then reacted with 3,3'-diaminobenzidine tetrahydrocholoride for visualization. After staining, the sheet was washed several times with water and dried.

Enzymatic deglycosylation of EA4 using N-glycanase – PNGase F (N-glycanase (EC 3.2.2.18), 25,000 units/mg of enzyme protein) was obtained from Boehringer-Mannheim (Germany). The enzyme of 10 units (10μ 1) was added to 1 nmol of EA4 (about 1 ml) in the HEPES buffer containing 50 mM NaCI, 20 mM KC1, 1.0 mM EDTA, 2.5% Triton X-100, 0,2% SDS and 1% 2-mercaptoethanol in final concentration. The mixture was incubated first at 5°C for 1 week and then transferred to 25°C to incubate for additional 1h. At appropriate intervals during the incubation, an aliquot was assayed for ATPase or ligand blots.

MALDI-TOF-MS - MALDI-TOF-MS spectra of EA4 were obtained using 2,5-dihydroxybenzoic acid as matrix and those for the mixtures of EA4 and PIN were obtained using a-cyano 4-hydroxycinnamic acid or 3,5-dimethoxy-4-hydroxy cinnapinic acid as matrix. Each μof sample (EA4, about 10 pmol; or the mixture, about 5 pmol EA4 and about 50 pmol PIN) was placed in a plastic bottle to which was added a two μ1 of matrix solution (saturated in acetone). The resultant solution was quickly transferred on a target (sample plate) and then allowed to crystallize in a refrigerator with spontaneous evaporation. Finally, the target was attached to a mass spectrometer. The MS spectra were measured on a TofSpec E mass spectrometer (MicroMass Co. Ltd., United Kingdom) in reflectron mode. Positive ions were generated by a pulsed nitrogen laser beam. The data were processed with a MassLynx program.

Surface plasmon resonance measurements - A carboxymethyl dextran lAsys cuvette (Affinity Sensors) was activated with 200 mM 1-ethy 1-3 (3-dimethylaminopropyi) carbodiimide (Pierce), 50 mM N-hydroxysuccinimide (Pierce) for 7 min and washed extensively with PBS (9). PIN at 27.38 μ.M was coupled to the activated cuvette in 10 mM sodium acetate buffer, pH 5.0, for 12 min at room temperature (330 arc seconds, 1.65 ng/mm^2). Uncoupled PIN was washed away with PBS and free amines blocked with 1 M ethanolamine (pH 8.5) for 3 min. A stable baseline was established for 10 min before data collection. All binding experiments were performed with EA4 over a range of concentrations from 2.2 to 8.8 ^m in volumes of 70 μ1. The binding surface was regenerated between binding measurements using 30 mM HC1 with no decrease in extent measurements over the duration of an experiment. Data were collected over 3-s intervals and analyzed using the FASTfit™ software provided with the lAsys instrument.

3. Result

Purification of EA4 and detection of glycoprotein by lectin binding -EA4 obtained by the rechoromatography on the YMC-Pack PROTEIN- RP column (the absorbance peak fraction with a retention time of 33 min) showed a single band by silver staining on SDS-PAGE (Fig. 1). The MALDI-TOF-MS spectrum of the EA4 contained a single peak at m/z 17,387 (Fig. 2). A smaller m/z peak on the MS spectrum (8,694) is probably a double charged ionized species of EA4. Thus, EA4 was applicable to lectin binding analysis.

We tested five different lectins to bind to the EA4 (Fig. 3). All five, SSA, DSA and Con A, and to a lesser extent AAL and RCA120, led to detection of the band. AAL and RCA120 are known to bind fucose and galactose residues, respectively. The latter RCA120 reaction is reduced by Sia α (2-6) Gal, the presence of which being indicated by SSA reaction.

The high intensity of the DSA and Con A reactions indicated that EA4 contained Gal β(l-4)GlcNAc units and trimannosyi core structures of Man α l-6(Man *a*

1-3) Man. Essentially the same lectin reactions were observed with the EA4 preparation obtained from the Sephadex G-25 filtration (data not shown).
Although the precise structure of the carbohydrate chains is uncertain until further analysis, EA4 was characterized as a glycoprotein.

Enzymatic deglycosylation of EA4- We conducted deglycosylation experiments to address the function of the carbohydrate moiety involved in the mechanism of time measurement. To cleave glycosyi chains, enzymes such as Endo H, Endo F and PNGase F were first applied to EA4 obtained by Sephadex G-25 filtration. Treatment of EA4 with the former two enzymes, however, resulted in no decrease in molecular mass and no decrease in Con A binding (data not shown). In contrast, treatment with PNGase F at 37°C for 20h completely abolished Con A binding to EA4 and resulted in a decrease in the EA4 molecular mass by approximately 2.5 kDa (Fig. 4). Since PNGase F is known to release proximal N-acetylglucosamine linked to Asn of peptides, the whole glycosyi chain may be cleaved. Irrespecitve of this effectiveness, the incubation condition for the deglycosylation was not applicable to the present timer-investigation. As discussed below, some modification of the deglycosylation condition was necessary.
The one time-transitory burst activation of EA4 shows a temperature dependency in solution (5, 10*)*. Without PIN, EA4 suddenly increased reaching maximal activity in about 7h at 25℃ (Fig. 5 and Ref.5) instead of taking about 2 weeks at 5°C (4, 7). Therefore, the temperature of 37℃ was too high and the duration of 20h was too long for the present experiment. Accordingly, various concentrations of deglycosylation by PNGase F were tested at various temperatures and with varying incubation times. Consequently, a temperature-incubation procedure was established: at 5°C for 1 week followed by additional 25°C for 1h in a reaction mixture of 1 nmol EA4 and 10 units PNGase F. By the incubation, EA4 abolished Con A binding and decreased molecular mass by 2.5 kDa (Fig. 4). Furthermore, EA4 did not complete the time measurement in that incubation period as mentioned in detail below.

Effect of enzymatic deglycosylation on EA4-timer - We studied whether the deglycosylated EA4 could measure the time interval. We had to carry the experiment with special care to the following two points. One of them is that the activation of PIN free-EA4 takes place virtually instantaneously at higher temperatures. For the time-run of EA4, as suggested previously, a one-day incubation at 5°C is equivalent to 30 min at 25°C (5). Therefore, an incubation for 1 week at 5°C followed by 1 h at 25°C was equivalent to one of 4.5h at 25°C. Additionally, about half a day at 5°C, equivalent to 0.25h at 25oC, was required for PIN removal and EA4 concentration by Centr icon-spin. Therefore, about 5h elapsed before the ATPase assay of deglycosylated EA4. Another point is that the coordination of clock-run in each EA4 molecule is disordered by the somewhat complicated treatments for deglycosylation. It was difficult to obtain data points to define the peak of EA4 appearance. Because of these two points, the ATPase activity of EA4 was measured by the method of one-time integration (5) instead of a time-course assay at intervals of 30 min. In the integration assay, amounts of liberated phosphate were detected during successive 3h intervals after 5h, 5-8h and 8-llh incubation. The 5-8h time span was expected to coincide with EA4 appearance and the 8-llh time span was expected to coincide with EA4 disappearance (Fig. 5).
As shown in Figure 6, while the amount of liberated phosphate was low during the incubation done between the last 8-llh, high amounts were detected between 5-8h. EA4 exhibited activity only between 5-8h after the onset of incubation, even though the activation period of each EA4 protein molecule should be very short, and the detected amount of phosphate was the integrated amount liberated during that period. It is worth noting that the high amounts were detected with both native and deglycosylated EA4 during the same 5-8h time span. The carbohydrate moiety may not directly contribute to the time measurement of EA4.

Significance of carbohydrate moiety on the PIN-regulation - To examine the function of the carbohydrates, in the next experiments PIN was added to EA4. A 76 µ1 PIN fraction of Centricon-3™ retentate (about 150 µg protein equivalent) was mixed with 40 *a* \ EA4 (about 0.8 µg protein) and assayed for ATPase activity. As shown in Figure 7, the activity of native EA4 during the 5-8h time span after incubation was strongly inhibited by PIN. This result is in accord with the previous finding that PIN inhibits EA4 (5, 7). On the other hand, no inhibition was observed when deglycosylated EA4 was mixed with PIN; deglycosylated EA4 exhibited essentially the same activity as without PIN. It is apparent that the carbohydrate moiety is indispensable for the execution of inhibitory activity by PIN.

Interaction between EA4 and PIN - PIN peptide was synthesized chemically and the mixture of the PIN and EA4 was analysed by MALDI-TOF-MS (Fig. 8). Since the Mr of PIN is 4,618 Da (6), Figure 8

demonstrates that EA4 forms an equimolar compex with PIN. A smaller m/z peak on the MS spectrum (14,266) is probably a by-product in the chemical synthesis of PIN. This is the first direct binding datum, and it has become appear ent that 1 mol of PIN is bound to 1 mol of EA4. To investigate further the interaction between EA4 and PIN, the binding affinity was measured by surface plasmon resonance. Binding of EA4 to immobilized PIN was measured over a range of EA4 concentrations (2.2-8.8 μM).

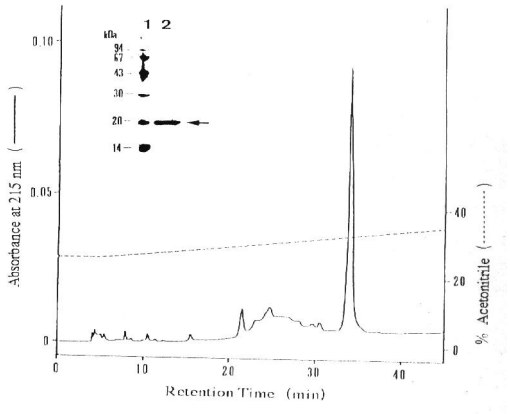

Fig. 1. Isolation of EA4 by RPC on YMC-Pack PROTEIN-RP column. Chromatographic conditions; 25% B for 5 min, 25-35% B linear gradient for 40 min at a flow rate of O.Sml/min, where solvent A was aqueous containing 0.05%(v/v) trifluoroacetic acid and solvent B was 100% acetonitrile containing 0.05% trifluoroacetic acid. Inset; SDS-PAGEof the isolated EA4 on 12.5% gel (lane 1, molecular mass standards; lane 2, isolated EA4; the arrow indicates the position of EA4; numbers indicate molecular mass on kDa).

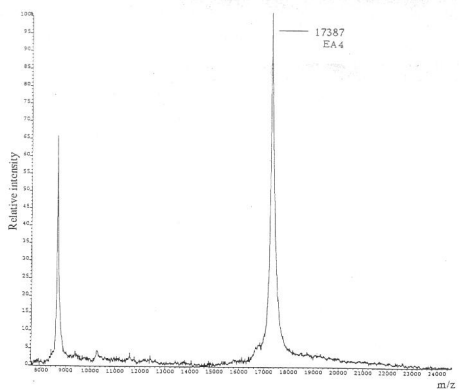

Fig. 2. MALDI-TOF-MS spectrum of EA4 isolated by the rechromatography on YMC-Pack PROTEIN-RP column. The spectrum was acquired in the reflectron mode using 2,5-dihydroxybenzoic acid crystals as matrix.

The association rate constant (K_a $4.370x10^3M^{-1}s^{-1}$) was obtained by plotting the measured K_{on} versus EA4 concentration (Fig. 9). The dissociation rate constant ($K_d 2.003x10^{-3}s^{-1}$) was obtained directly from dissociation experiments. These values were used to calculate a K_D of 460nM for the EA4-PIN interaction. The equilibrium constant in the order of 10^{-7}M provides significant information regarding the timer regulation as discussed below.

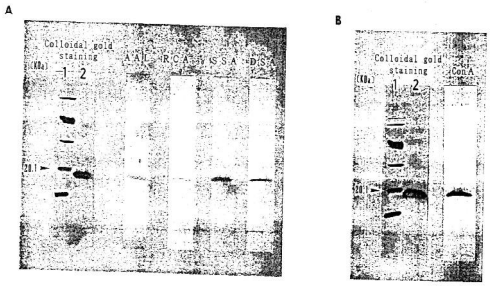

Fig. 3. Lectin blot analysis of EA4. EA4 was subjected to SDS-PAGE and electrophoretically transferred to PVDF membrane, which was then cut into strips. The strips were exposed to biotinyl-AAL, RCA120, SSA, DSA (panel A) and Con A (panel B), followed by reaction with avidin-biotinyl-peroxidase complexes and staining with 3,3-diaminobenzidine. The strip containing the moled uar-weight makers (1) and one of the sample strips (2) were stained in colloidal gold (protein standards, the same molecular masses as shown in the Fig. 1).

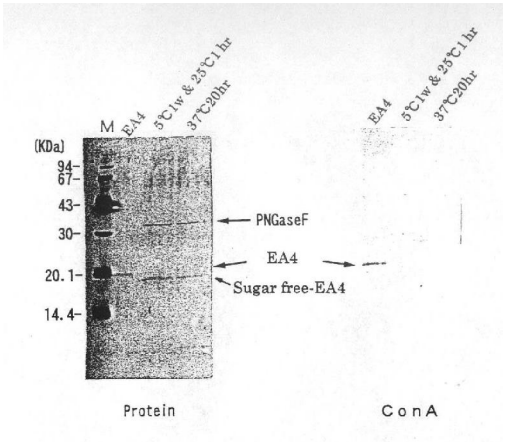

Fig. 4. Western blots of native and deglycosylated EA4 after treatment with PNGase F at 37°C for 20 hr or 5°C for 1 week followed by additional 25°C for 1h. Proteins were stained by colloidal gold. M, protein standards. Numbers indicate molecular mass on kDa. Glycoprotein was detected with biotinyl-Con A, followed by reaction with avidin-biotinyl-peroxidase complexes and staining with 3,3'-diaminobenzidine.

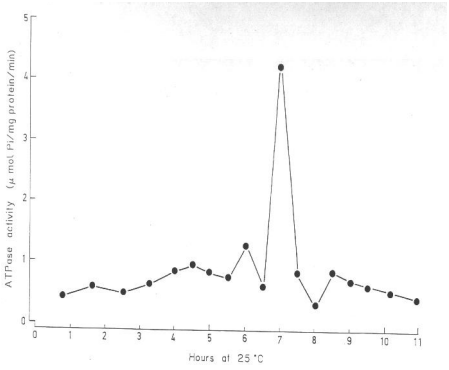

Fig. 5. Time-interval activation of EA4 in ATPase activities during incubation at 25°C for 11h. The EA4 preparation was freed from PIN fraction by Centr icon-spin (see details in the text) and the ATPase activities were determined at about 30 min intervals during the incubation. The numbers on the abscisa indicate the time elapsed after the Sephadex G-25 step of the EA4 preparation. (From reference 5.)

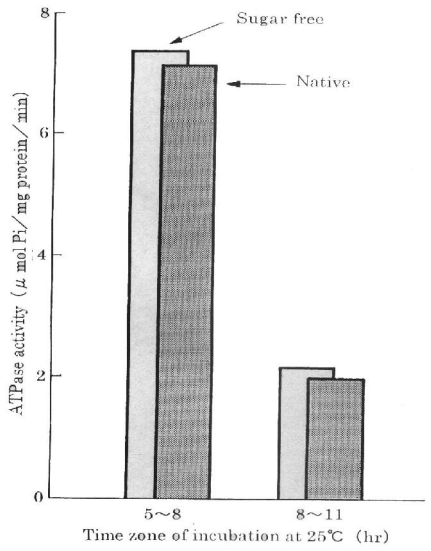

Fig. 6. Changes in ATPase activities measured by the method of one-time integration during incubation of sugar-free EA4 at 25°C. Sugar-free EA4 was obtained by PNGase F treatment. ATPase assays were carried at 25°C during successive 3h time intervals. The time intervals were expressed by the equivalent time elapsed after the Sephadex G-25 step of the EA4 preparation (the same applies to the Fig. 7).

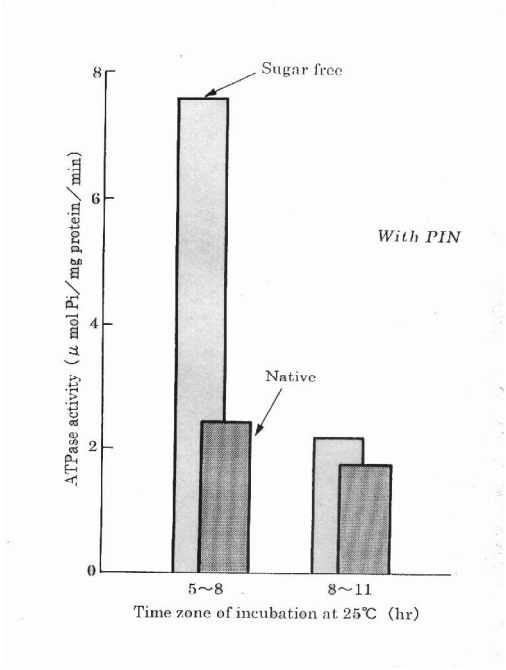

Fig. 7. Significance of sugar chain in PIN-effect on the time-interval activation of EA4. EA4 was mixed with PIN and the ATPase activity was assayed by the method of one-time integration.

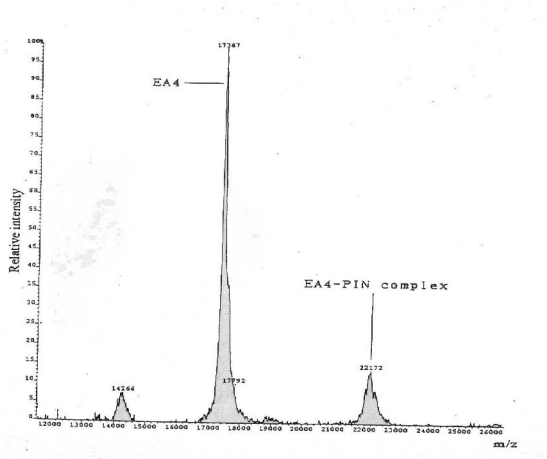

Fig. 8. MALDI-TOF-MS spectrum used for the determination of EA4-PIN complex. Mixture of EA4 and PIN was deposited on a thin layer of α-cyano 4-hydroxycinnamic acid crystals and the spectrum of them was acquired in the reflectron mode.

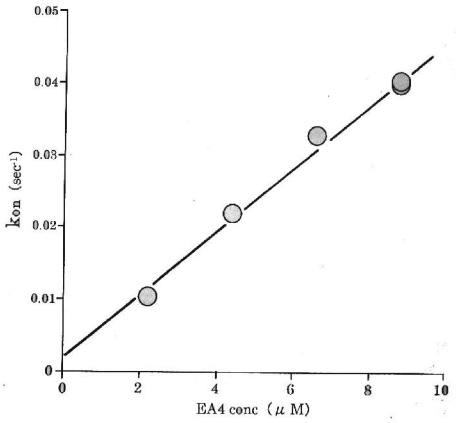

Fig. 9. Affinity measurement of EA4 and PIN. The binding affinity of EA4 and PIN was measured by surface plasmon resonance. The PIN was immobilized on the surface of the lAsys cuvette, and the immobilized PIN was incubated with EA4 over a range of concentrations (2.2-8.8 μM).Shown is a representative plot from three experiments.

4. Discussion

EA4 may have the possible capability of measuring a time interval in accordance with development (4). PIN seems to regulate time measurement (6, 7). PIN may also be involved in the mechanism by which the EA4 timer operates only in cold. The interaction between EA4 and PIN is important in the timer-mechanism, and the results of this study provide evidence that 1) PIN binds to EA4 forming an equimolar complex, 2) EA4 has carbohydrate moieties and 3) the carbohydrate is essential for the assembly of a high affinity PIN-binding site within the timer motif of the EA4 structure. Although the exact mechanisms of time-mesurement and time-regulation must be considered speculative until further analysis is carried out, it is revealed that formation of a regulatory substructure is involved in those mechanisms.

The equilibrium constant for the EA4-PIN interaction provides significant information regarding the mechanisms. The equilibrium constant is within the physiological concentration range for these peptides. Besides, the constant in the 10^7 molar range means that the affinity is not too high and, at the same time, not too low. These are consistent with the hypothesis that the association-dissociation inter conversion may be involved in the regulatory mechanism in the cell. One possible explanation is that EA4 in eggs may originally be in complex with PIN and that environmental signals may induce the dissociation of the complex to localize EA4 and PIN. The dissociation could constitute the cue for time measurement to EA4 activity burst, which in turn initiates new developmental programs. Whether or not the association and dissociation is involved in the regulatory mechanism is now under investigation in this laboratory.

The result of lectin binding analysis indicates that N-linked oligosaccharides may be involved. Based on the detected carbohydrate compositions, the lowest molecular mass of the oligosaccharides is estimated to be about 2.5 kDa, although no definitive strucuture has been established. PNGase F is an endoglycosidase known to release proximal N-acetylglucosamine linked to Asn of peptide. Treatment of EA4 with the PNGase F resulted in a decrease in molecular mass of about 2.5 kDa. In addition, only one glycosylation site (Asn-Ile-Thr) has been detected in EA4 (unpublishied results). Taken together, these findings suggest EA4 may contain a single oligosaccharide chain. Determination of the precise structure of the oligosaccharide and EA4, itself, is critical in the timer investigation and are now in progress.

Eggs of animals seem to undergo carefully timed sequence of events controlled by endogenous timing mechanisms. To control when genes act, cells must be able to measure time. Examples of a timed event in development are provided by the regulated apoptosis at the onset of gastrulation'in *Xenopiis* embryos (77, 72, 73, 14). The timing and execution of the maternal cell death program is set at fertilization and is independent of the types of stress applied on cell cycle progression or on *denovo* protein synthesis. There are long-term mechanisms which trigger the events at the proper time. EA4 might be one of candidates for the timer-protein as a Time Interval Measuring Enzyme (TIME).Although many scientists working to understand diapauses by using molecular biology techniques Gkouvitsqs, et al (2009)in Secamia creatica ,also Qiu, et al (2007) in crustacean, Kion andDenlinger (2009) in Culex pipiens , but this system of analysis is still the cheaper and saving time, to understand the programmers of diapouse, Also with emphases on Egyptian secamia creatica races in Egyptian phona.

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