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Carbohydrate Moiety of Time-Interval Measuring Enzyme Regulates Time Measurement through Its Interaction with Time-Holding Peptide PIN[1]

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An ATPase called EA4 seems to measure time as a diapause-duration timer in the seasonal cycle of the silkworm, *Bombyx mori*. A peptide named PIN seems to regulate the time measurement of EA4. We characterize the EA4 as the first step to analyse its interaction with PIN. Matrix-assisted laser desorption/ionization–time of flight–mass spectrometry shows EA4 forms an equimolar complex with PIN. The binding affinity of EA4 for PIN is about 460 nM, as measured by surface plasmon resonance. Western blot analysis of EA4 with a variety of biotinylated lectins suggests that EA4 is a glycoprotein containing *N*-linked oligosaccharide. On 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.

Key words: ATPase, *Bombyx mori*, glycoprotein, TIME-EA4, timer protein.

Biological systems that measure and mark elapsed time may be involved in the accurate timing of developmental events in cells (*1*). 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 for ensuring that key reactions occur at certain times, like an alarm clock. Many important contributions have been made as to the identification of such time-measuring mechanisms. Regarding 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*, *3*). But we have comparatively few clues as to how cells are able to measure elapsed time through a long-term interval timer type of a biological clock. Recently, an ATPase called EA4 was found to possibly have the ability to measure time as a diapause-duration timer in the seasonal cycle of the silkworm, *Bombyx mori* (*4*, *5*).

The EA4 of *Bombyx* diapause eggs exhibits one-time transitory burst activation during the chilling of eggs to terminate the diapause. It is noteworthy that the activation is also observed *in vitro*. The sudden increase in EA4 activity *in vitro* was equivalent to that observed *in vivo* and was coincident with the chilling period that is known to be indispensable for diapause termination. An *in vivo* and *in vitro* combination experiment demonstrated that the *in vitro* activation of EA4 occurred at the same rate as *in vivo* 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 activation of the enzyme inhibited but also the clock-run of EA4 was 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 a time-interval and the question of how EA4 activation is related to the resumption of embryogenesis remain to be resolved. In this context, it is crucial that the time-interval activation of EA4 be accomplished after Sephadex G-25 filtration on EA4 purification (*4*, *5*). Certain considerations regarding the Sephadex-results form the

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Abbreviations: AAL, *Aleuria aurantia* lectin; ConA, concanavalin A; DSA, *Datura stramonium* agglutinin; Endo F, endo-β-*N*-acetylglucosaminidase F; Endo H, endo-β-*N*-acetylglucosaminidase H; Gal, galactose; GlcNAc, *N*-acetylglucosamine; MALDI-TOF-MS, matrix-assisted laser desorption ionization–time of flight–mass spectrometry; Man, mannose; PBS, phosphate-buffered saline; PNGaseF, peptide-*N*-glycosidase F; RCA, *Ricinus communis* agglutinin; RPC, reversed phase chromatography; Sia, sialic acid; SSA, *Sambucus sieboldiana* agglutinin.

basis for the design of the following experiment.

EA4 is eluted later than the void volume fraction on a Sephadex G-25 column, even though its molecular mass is about 20 kDa (*4*). Thus, a Sephadex column acts as a means of not gel-filtration but affinity chromatography. This suggests that a significant proportion of the EA4-mass may comprise carbohydrate structures, which contribute to the delayed elution from a Sephadex column. This suggests that the EA4 carbohydrate moiety is involved in the mechanism of time measurement. Therefore, the glycoprotein characterization of EA4 is of importance in 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 interaction may play a central role in the EA4 timer.

MATERIALS AND METHODS

Preparation of EA4 and PIN—C108(old) *Bombyx* silkworm diapuase eggs were used. Eggs laid within 3 h were collected at 25°C to obtain synchronous egg batches. Two days (50 h) after oviposition, the eggs were washed with cold acetone and then EA4 was prepared. The preparation procedures are described in detail in a previous paper (*5*), and included the production of acetone powder of the eggs, heat treatment at 85°C, precipitation with 80% saturated ammonium sulfate, and gel filtration through a Sephadex G-25 column, EA4 being eluted later than the void volume fraction. The collected EA4 fractions were cleared of contaminating PIN by Centricon-10™ centrifugation (Amicon, Lexington, USA). PIN removal was accomplished by repeated dilution and filtration 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 NaCl, 20 mM KCl, and 1.0 mM EDTA. Unless otherwise noted, all preparation procedures were conducted in a cold room (4°C) or an ice-water bath.

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

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

Incubation of EA4 and ATPase Assaying—ATPase assaying of the EA4 preparation was performed at 25°C for 30 min or 3 h in sterilized silicon-coated test tubes (*5*); the reaction HEPES buffer contained 50 mM NaCl, 20 mM KCl, 1.0 mM EDTA, and 100 μg/ml salmon testes DNA. The time of EA4 activity-appearance was expressed as the time that had elapsed after the Sephadex G-25 step of the EA4 preparation as reported previously (*5*).

Purification of EA4 for Lectin-Binding—EA4 was further purified for lectin binding. First, the EA4 preparation described above was separated and desalted using disposable cartridges packed with silica bonded to C_{18} hydrocarbon (Sep-Pak C_{18} cartridges; Waters Associates). Each 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 100 μl Milli Q water containing 0.05% TFA for application to a 4.6 mm × 25 cm (5 μm) YMC-Pack PROTEIN-RP (YMC, Kyoto) reverse-phase column in 0.05% TFA in Milli Q water (solvent A). The column was eluted with a stepwise gradient generated from solvents A and B (0.05% TFA in 100% acetonitrile) at the flow rate of 1 ml/min, with the following time course: solvent A (5 min), 0→30% B (5 min), 30% B (5 min), and 30→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 100 μl 25% acetonitrile in 0.05% TFA for reapplication to YMC-Pack PROTEIN-RP. The second column was eluted at the flow rate of 0.8 ml/min, using a gradient of 25% B (5 min) and 25→35% B (40 min). The absorbance peak of 33 min was collected and used for the following lectin blot analysis. All HPLC procedures were performed at room temperature.

Electrophoretic Transfer of Proteins to PVDF Sheets and Lectin-Binding for Glycoprotein Detection—SDS-PAGE was performed in 1-mm-thick slabs with a RAPIDAS Mini-Slab Electrophoresis Cell (ATTO, Tokyo) by the procedure of Laemmli (*8*). Briefly, the separating gels contained 12.5% (w/v) acrylamide, 0.5% *NN'*-methylenebisacrylamide, 0.1% SDS, and 375 mM-Tris/HCl, pH 8.8; and the stacking gels contained 4.5% (w/v) acrylamide, 0.18% *NN'*-methylenebisacrylamide, 0.1% SDS, and 125 mM Tris/HCl, pH 6.8. The running buffer was 25 mM Tris/19.2 mM glycine/0.1% SDS, pH 8.3. After each run, the proteins were either transferred to a polyvinylidene difluoride membrane (see below), or fixed and stained with silver (Silver Stain Kit Wako; Wako, Osaka).

For the electrophoretic transfer of proteins to polyvinylidene difluoride membranes (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) containing electrode buffer (20% methanol/100 mM Tris/192 mM glycine, pH 8.3). 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 molecular-weight markers were then stained in colloidal gold (LECTIN SENSOR Honen; Honen, Tokyo). 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 I (10 mM Tris/HCl, 0.053% Tween 20, 1% NaCl, pH 7.4) four times for 15 min each time and then incubated with biotinylated lectins (LECTIN SENSOR Honen) for 80 min (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 a horseradish peroxidase–avidin solution (HRP-avidin;

Honen). The treated sheet was washed four times with blocking buffer I, and 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, 10 units (10 μl), was added to 1 nmol of EA4 (about 1 ml) in the HEPES buffer containing 50 mM NaCl, 20 mM KCl, 1.0 mM EDTA, 2.5% Triton X-100, 0.2% SDS, and 1% 2-mercaptoethanol, final concentrations. The mixture was incubated first at 5°C for 1 week and then transferred to 25°C for incubation for an additional 1 h. At appropriate intervals during the incubation, an aliquot was subjected to ATPase assaying or ligand blotting.

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

Surface Plasmon Resonance Measurements—A carboxymethyl dextran IAsys cuvette (Affinity Sensors) was activated with 200 mM 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (Pierce), 50 mM *N*-hydroxysuccinimide (Pierce) for 7 min, and then washed extensively with PBS (*9*). PIN at 27.38 μM 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/mm²). 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 in the concentration range of 2.2 to 8.8 μM in a volume of 70 μl. The binding surface was regenerated between binding measurements using 30 mM HCl with no decrease in the extent measurements over the duration of an experiment. Data were collected at 3-s intervals and analyzed using the FASTfit™ software provided with the IAsys instrument.

RESULTS

Purification of EA4 and Detection of Glycoprotein by Lectin Binding—EA4 obtained on rechromatography on the YMC-Pack PROTEIN-RP column (the absorbance peak fraction with a retention time of 33 min) gave a single band on silver staining on SDS-PAGE (Fig. 1). The MALDI-TOF-MS spectrum of EA4 contained a single peak at m/z 17,387 (Fig. 2). A smaller m/z peak in 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 as to binding to 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 RCA120 reaction is reduced by Siaα(2-6) Gal, the presence of which is indicated by the SSA reaction. The high intensity of the DSA and Con A reactions indicated that EA4 contained Galβ(1-4)GlcNAc units and trimannosyl core structures of Manα1-6(Manα1-3) Man. Essentially the same lectin reactions were observed with the EA4 preparation obtained on Sephadex G-25 filtration

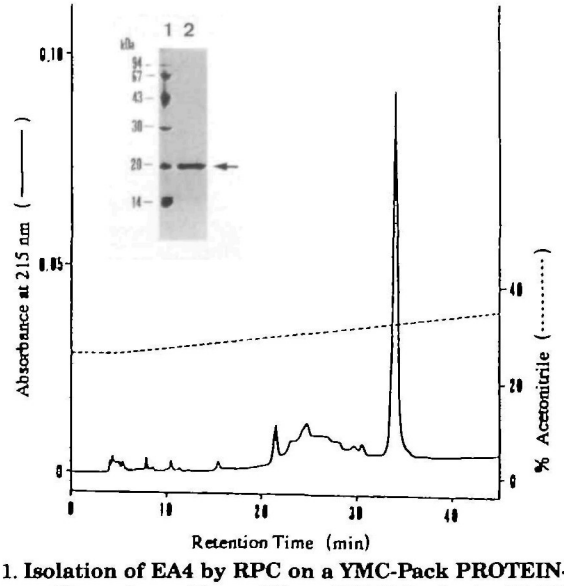

Fig. 1. **Isolation of EA4 by RPC on a YMC-Pack PROTEIN-RP column.** Chromatographic conditions: 25% B for 5 min, 25–35% B linear gradient for 40 min at the flow rate of 0.8 ml/min, where solvent A was aqueous containing 0.05% (v/v) trifluoroacetic acid and solvent B was 100% acetonitrile containing 0.05% trifluoroacetic acid. Inset: SDS-PAGE of the isolated EA4 on a 12.5% gel (lane 1, molecular mass standards; lane 2, isolated EA4; the arrow indicates the position of EA4; numbers indicate molecular masses in kDa).

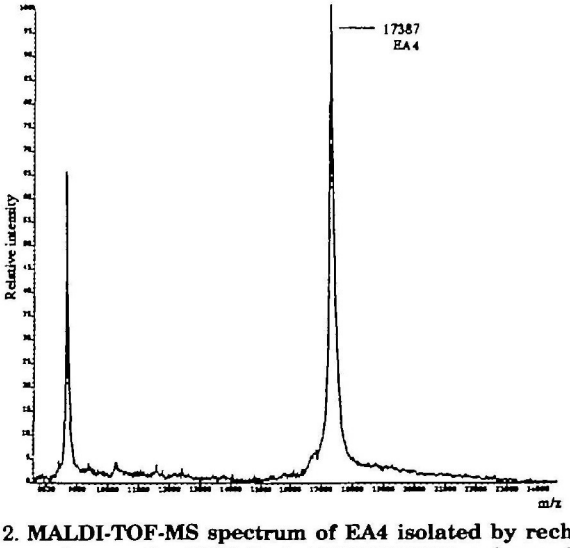

Fig. 2. **MALDI-TOF-MS spectrum of EA4 isolated by rechromatography on the YMC-Pack PROTEIN-RP column.** The spectrum was acquired in the reflectron mode with 2,5-dihydroxybenzoic acid crystals as the matrix.

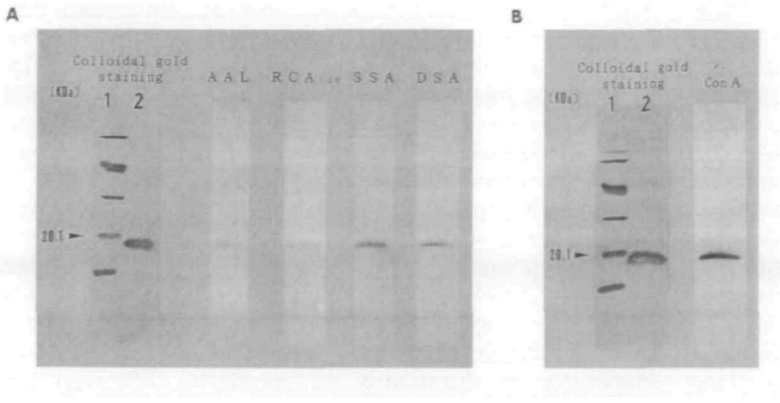

Fig. 3. **Lectin blot analysis of EA4.** EA4 was subjected to SDS-PAGE and then electrophoretically transferred to a PVDF membrane, which was then cut into strips. The strips were exposed to biotinyl-AAL, RCA120, SSA, DSA (panel A), and Con A (panel B), followed by reaction with avidin-biotinyl-peroxidase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

(data not shown).

Although the precise structure of the carbohydrate chains will remain 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 glycosyl 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 20 h completely abolished the 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 glycosyl chain may be cleaved. Irrespective of this effectiveness, the incubation conditions for the deglycosylation were not applicable to the present timer investigation. As discussed below, some modification of the deglycosylation conditions was necessary.

The one time-transitory burst activation of EA4 shows temperature dependency in solution (*5*, *10*). Without PIN, EA4 suddenly increased, the maximal activity being reached in about 7 h at 25°C (Fig. 5 and Ref. *5*) instead of about 2 weeks at 5°C (*4*, *7*). Therefore, the temperature of 37°C was too high and the duration of 20 h was too long for the present experiment. Accordingly, various concentrations for deglycosylation by PNGase F were tested at various temperatures and with varying incubation times. Consequently, a temperature-incubation procedure was established: 5°C for 1 week followed by 25°C for an additional 1 h with a reaction mixture of 1 nmol EA4 and 10 units PNGase F. With the incubation, the Con A binding to EA4 was abolished and its molecular mass decreased 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 the EA4-Timer—We examined whether or not the deglycosylated EA4 could measure a time interval. We had to carry the experiment with special care as 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, 1-day incubation at 5°C is equivalent to 30 min at 25°C (*5*). Therefore, incubation for 1 week at 5°C followed by 1 h at 25°C was equivalent to one of 4.5 h at 25°C. Additionally, about half a day at 5°C, equivalent to 0.25 h at 25°C, was required for PIN removal and EA4 concentration by Centricon-spin. Therefore, about 5 h elapsed before the ATPase assaying of deglycosylated EA4. Another point is that the coordination of the 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, the amounts of liberated phosphate were determined during successive 3 h intervals after 5, 5–8, and 8–11 h incubation. The 5–8 h time span was expected to coincide with EA4 appearance and the 8–11 h time span was expected to coincide with EA4 disappearance (Fig. 5).

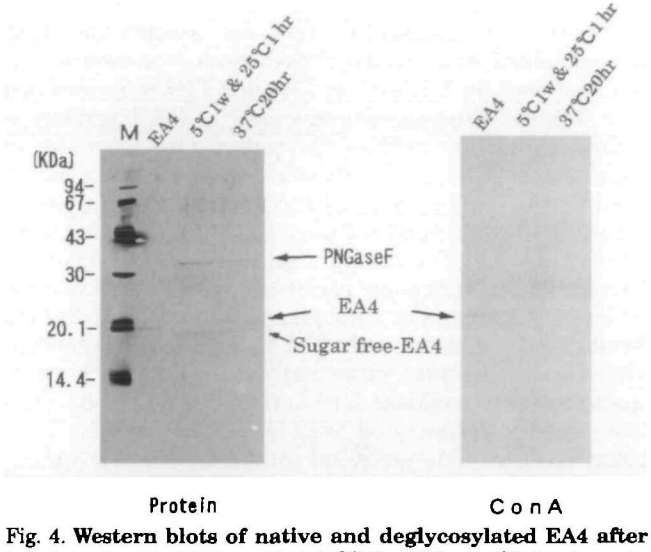

Fig. 4. **Western blots of native and deglycosylated EA4 after treatment with PNGase F at 37°C for 20 h or 5°C for 1 week, followed by 25°C for an additional 1 h.** Proteins were stained with colloidal gold. M, protein standards. Numbers indicate molecular masses in kDa. Glycoprotein was detected with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

As shown in Fig. 6, while the amount of liberated phosphate was low during the incubation performed between 8–11 h, high amounts were detected between 5–8 h. EA4 only exhibited activity between 5–8 h after the onset of incuba-

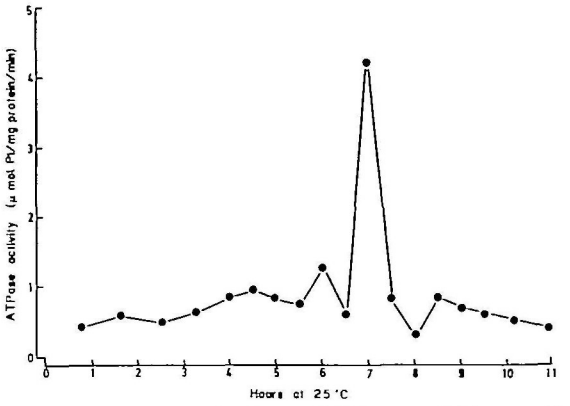

Fig. 5. **Time-interval activation of EA4 as to ATPase activity during incubation at 25°C for 11 h.** The EA4 preparation was freed from PIN by Centricon-spin (details in the text), and the ATPase activity was determined at about 30 min intervals during the incubation. The numbers on the abscissa indicate the time that had elapsed after the Sephadex G-25 step of the EA4 preparation (from Ref. 5).

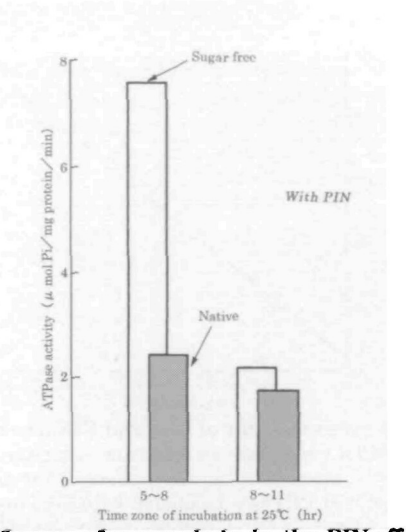

Fig. 7. **Significance of sugar chain in the PIN-effect on the time-interval activation of EA4.** EA4 was mixed with PIN and then ATPase activity was assayed by the method of one-time integration.

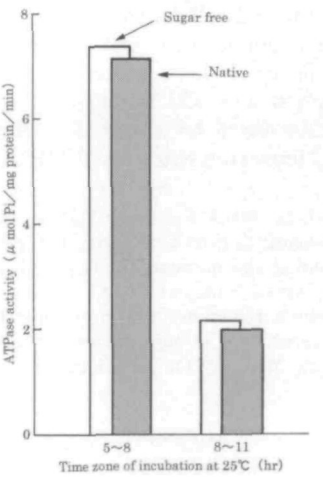

Fig. 6. **Changes in ATPase activity measured by the method of one-time integration during incubation of sugar-free EA4 at 25°C.** Sugar-free EA4 was obtained by PNGase F treatment. ATPase assays were carried at 25°C after successive 3 h time intervals. The time intervals are expressed as the equivalent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

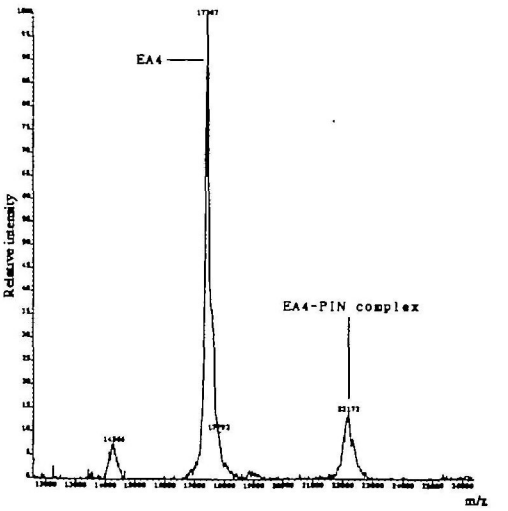

Fig. 8. **MALDI-TOF-MS spectrum used for determination of the EA4-PIN complex.** A mixture of EA4 and PIN was deposited on a thin layer of α-cyano 4-hydroxycinnamic acid crystals and then a spectrum was acquired in the reflectron mode.

tion, 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 the period. It is noteworthy that high amounts were detected with both native and deglycosylated EA4 during the same 5–8 h time span. The carbohydrate moiety may not directly contribute to the time measurement of EA4.

Significance of the Carbohydrate Moiety as to PIN-Regulation—To examine the function of the carbohydrates, in the next experiments PIN was added to EA4. 76 μl PIN fraction of the Centricon-3™ retentate (about 150 μg protein equivalent) was mixed with 40 μl EA4 (about 0.8 μg protein), followed by assaying for ATPase activity. As shown in Fig. 7, the activity of native EA4 during the 5–8 h 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 inhibitory activity of PIN.

Interaction between EA4 and PIN—The PIN peptide was synthesized chemically, and a mixture of PIN and EA4 was analysed by MALDI-TOF-MS (Fig. 8). Since the molecular mass of PIN is 4,618 Da (*6*), Fig. 8 demonstrates that EA4 forms an equimolar complex with PIN. A smaller m/z peak in 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 apparent that 1 mol of PIN is bound to 1 mol of EA4. To further investigate the interaction between EA4 and PIN, the binding affinity was measured by means of surface plasmon resonance. The binding

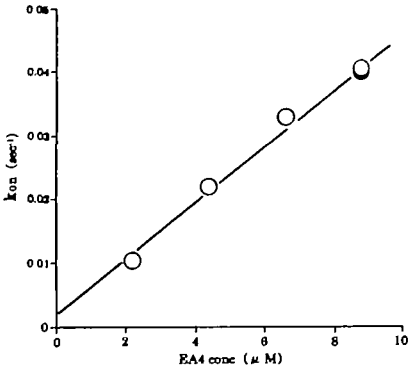

Fig. 9. **Affinity measurement of EA4 and PIN.** The binding affinity of EA4 and PIN was measured by means of surface plasmon resonance. PIN was immobilized on the surface of the IAsys cuvette, and the immobilized PIN was incubated with EA4 over a range of concentrations (2.2–8.8 μM). A representative plot of three experiments is shown.

of EA4 to immobilized PIN was measured over a range of EA4 concentrations (2.2–8.8 μM). The association rate constant (k_a 4.370×10^3 M^{-1} s^{-1}) was obtained by plotting the measured k_{on} *versus* the EA4 concentration (Fig. 9). The dissociation rate constant (k_d 2.003×10^{-3} s^{-1}) was obtained directly through dissociation experiments. These values were used to calculate a K_D of 460 nM for the EA4-PIN interaction. The equilibrium constant of the order of 10^{-7} M provides significant information regarding the timer regulation, as discussed below.

DISCUSSION

EA4 may possibly have the ability to measure 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 only operates in the cold. The interaction between EA4 and PIN is important in the timer-mechanism, and the results of this study provide evidence that (i) PIN binds to EA4, forming an equimolar complex, (ii) EA4 has carbohydrate moieties, and (iii) 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-measurement and time-regulation must be considered speculative until further analysis is carried out, it was revealed that the formation of a regulatory substructure is involved in these 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 interconversion may be involved in the regulatory mechanism in the cell. One possible explanation is that EA4 in eggs may originally be in a 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 through the 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 results of lectin binding analysis indicate 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 structure has been established. PNGase F is an endoglycosidase known to release proximal *N*-acetylglucosamine linked to Asn of peptides. Treatment of EA4 with PNGase F resulted in a decrease in its molecular mass of about 2.5 kDa. In addition, only one glycosylation site (Asn-Ile-Thr) has been detected in EA4 (unpublished results). Taken together, these findings suggest that EA4 may contain a single oligosaccharide chain. Determination of the precise structures of the oligosaccharide and EA4, itself, is critical for timer investigation, and this is now in progress.

Eggs of animals seem to undergo a 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 *Xenopus* embryos (*11–14*). The timing and execution of the maternal cell death program is set at fertilization and is independent of the type of stress applied on cell cycle progression or *de novo* protein synthesis. There are long-term mechanisms which trigger the events at the proper time. EA4 might be a candidate timer-protein as a Time Interval Measuring Enzyme (TIME).

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