Chemical Structure of Nuclear Proteins Which Are Phosphorylated during Meiotic Maturation of Starfish Oocytes†

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Received December 2, 1999; Revised Manuscript Received February 23, 2000

ABSTRACT: Oocytes of the starfish, *Asterina pectinifera*, are arrested at the G2 phase of meiosis I and possess a prominent germinal vesicle in which maternal stores of nuclear proteins which are destined for use primarily by the early embryo are stored. Germinal vesicle breakdown and subsequent oocyte maturation is triggered by activation of the $p34^{cdc2}$/cyclin B complex, which is present as the preform in the cytoplasm. The aim of the present study was to identify and biochemically characterize in vivo substrates of the kinase. Two nucleic acid binding nuclear proteins designated NAAP1 and NAAP2 were found, both of which contain 345 amino acid residues with pI 3.6 and which serve as substrates. The only difference between the two proteins was in the primary amino acid sequence at position 51, which is Asn in NAAP1 but Thr in NAAP2. NAAPs are phosphorylated in vivo during oocyte maturation but not at the meiotic G_2 stage. NAAPs are phosphorylated in vitro by the cdc2 kinase on the same site as in vivo. Although there are other evolutionarily conserved consensus sequences for phosphorylation by mitotically active cdc2 kinase in NAAPs and NAAP-derived fragments containing the sequences were efficiently phosphorylated in vitro, these sites in the intact NAAPs were not phosphorylated either in vivo or in vitro. These results suggest that the tertiary structure of NAAPs affects the target specificity of the cdc2 kinase.

Starfish oocytes represent ideal models for investigations of meiotic cell cycle progression, since they can be obtained in large numbers in a state of meiotic G_2 arrest (*1*). The oocytes contain an intact germinal vesicle (a large nucleus) with a 4N chromosome set. Meiotic maturation is initiated by the addition of 1-methyladenine, the natural maturation-inducing substance (*1*), which causes synchronous germinal vesicle breakdown and subsequent oocyte maturation thus rendering the oocytes fertilizable. Meiotic oocyte maturation can also be initiated via microinjection of maturation-promoting factor (MPF).[1] MPF, which was discovered in starfish by Kishimoto and Kanatani (*2*), was purified and characterized by Labbé et al. (*3*). MPF has been identified as the cdc2 protein kinase, a stoichiometric complex between cyclin B and $p34^{cdc2}$ (*4*). Starfish oocytes which have been arrested in the G_2 phase of meiosis I contain a $p34^{cdc2}$/cyclin B complex as the preMPF, which is maintained as the inactive form by phosphorylation of Thr-14 and Tyr-15 residues of its $p34^{cdc2}$ subunit (*3*). Dephosphorylation at these residues by phosphatases such as Cdc25 phosphatase activates the $p34^{cdc2}$/cyclin B complex and triggers exist from G_2 arrest (*5*).

Many of the cellular reorganizations which occur at the onset of mitosis are triggered, either directly or indirectly, by the activation of the $p34^{cdc2}$ protein kinase. However, although the mechanisms leading to the activation of $p34^{cdc2}$/cyclin B are reasonably well understood, little attention has been paid to its substrates in starfish oocytes.

The aim of the present study was to obtain substrates of $p34^{cdc2}$/cyclin B from starfish (*Asterina pectinifera*) oocytes. Two related nuclear proteins which are capable of binding double-stranded (ds) RNA, dsDNA, and other nucleic acids, were detected in the germinal vesicle and were tentatively designated NAAP1 and NAAP2. Those compounds were purified to homogeneity, and their primary amino acid sequence determined, and posttranslational modifications characterized. The only difference which was detected in the primary amino acid sequence between NAAPs 1 and 2 was at position 51, in which the residue in NAAP1 is Asn whereas that in NAAP2 is Thr. A conspicuous feature of

† This work was supported, in part, by a Grant-in-Aid for scientific research from the Ministry of Education, Science, Sports and Culture, Japan.

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[1] Abbreviations: ASW, artificial seawater; BSA, bovine serum albumin; ds, double-stranded; HA, hydroxylapatite; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MPF, maturation-promoting factor; MS, mass spectrometry; MS/MS, tandem mass spectrometry; ss, single-stranded; PAGE, polyacrylamide gel electrophoresis; RP, reversed-phase; TFA, trifluoroacetic acid; TOF, time-of-flight.

10.1021/bi992759x CCC: $19.00
Published on Web 05/05/2000

these proteins is that they are phosphorylated in vitro by the $p34^{cdc2}$/cyclin B complex or other protein-serine/threonine kinases which are present in immature and maturing oocytes. However, in vivo, NAAPs are not substantially phosphorylated at the meiotic G_2 stage, but they are phosphorylated during meiotic maturation. These results indicate that NAAs are in vivo substrates of the $p34^{cdc2}$/cyclin B complex, which function during starfish oocyte maturation.

MATERIALS AND METHODS

Preparation and Labeling of Oocytes. Immature oocytes and maturing oocytes obtained 15 min after the initiation of 1×10^{-6} M 1-methyladenine treatment (*6*) were individually incubated in artificial seawater (ASW) containing [^{35}S]-methionine (20 μCi/mL), streptomycin sulfate (5 mg/mL), and penicillin G (50 μg/mL) at 20 °C for 30 min. Labeled oocytes were washed three times in ASW before they were lysed as described below.

Cultivation of Embryos and Subcellular Fractionation. Embryos were cultured in ASW containing streptomycin sulfate (5 mg/mL) and penicillin G (50 μg/mL), and their nuclear and cytosolic fractions were prepared as described previously (*7*).

Preparation and Purification of NAAPs. All purification steps were performed at 4 °C unless otherwise indicated. A protein assay was carried out at each purification step using the method of Lowry et al. (*8*) with bovine serum albumin (BSA) as a standard. Oocytes, sedimented by hand centrifugation (packed volume: 50 mL), were homogenized in 6 vol of chilled buffer A, which is composed of 20 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol. Homogenates were centrifuged for 20 min at 10000*g*, and the supernatant was collected. The supernatant was diluted with 2 volumes of buffer A, and the mixture was loaded on a DEAE-cellulose (Whatman) column (2.5 $\times$ 7.5 cm) which had been preequilibrated with buffer A. After being loaded, the column was washed with 600 mL of buffer A which contained 0.2 M NaCl and NAAPs were then eluted with a linear gradient (600 mL) of 0.2−0.4 M NaCl in buffer A with 30 mL fractions being collected. An aliquot (100 μL) of each fraction was dialyzed against buffer A and the dialysate was used for affinity binding with dsRNA as described below. To determine the fractions which contained in vitro substrates of a kinase(s), a 5 μL aliquot of each fraction was used for phosphorylation assay as described below. Fractions containing kinase substrates or dsRNA-binding activity were combined, and solid ammonium sulfate was added to the solution to 80% (w/v) saturation. The resulting precipitate was dissolved in 0.3 M sodium phosphate buffer (pH 7.2). The solution was then loaded onto a hydroxylapatite (HA, Bio-Rad) column (13 $\times$ 22 mm), which had been preequilibrated with 0.3 M sodium phosphate buffer (pH 7.2). After being loaded, the column was washed with 20 mL of 0.3 M sodium phosphate buffer (pH 7.2) and NAAPs were eluted with 18 mL of 0.4 M sodium phosphate buffer (pH 7.2) with 3 mL fractions being collected. Fractions which contained kinase substrates or dsRNA-binding activity were combined and concentrated by ultrafiltration using Ultra-free CL (Millipore). The concentrate was diluted with 0.1% trifluoroacetic acid (TFA), and the solution was chromatographed by reversed-phase (RP) high performance liquid chromatography (HPLC) using a μBondasphere C4−300 Å (Millipore) column (3.9 $\times$ 150 mm). NAAPs 1 and 2 were separated using a linear gradient of 30−100% acetonitrile (1%/min) in 0.1% TFA at a flow rate of 0.5 mL/min. UV (OD at 230 nm)-absorbing fractions were then dried in vacuo.

Phosphorylation. Samples were incubated in 20 μL of the reaction mixture which contained buffer B, which was composed of 25 mM Tris-HCl (pH 7.8), 2.5 mM magnesium acetate, and 50 mM KCl, and 2 μCi of [γ-^{32}P]ATP (25 μM, Amersham). The reaction was carried out at 20 °C for 10 min. For phosphorylation by cdc2 kinase, a reaction was carried out at 25 °C for 30 min in 20 μL of buffer C, which was composed of 80 mM sodium β-glycerophosphate (pH 7.3), 15 mM $MgCl_2$, 20 mM EGTA, 1 mM dithiothreitol, 100 mM KCl, and 100 mM sucrose, containing the $p34^{cdc2}$/cyclin B complex which had been purified from maturing *A. pectinifera* oocytes (*9*). For metabolic labeling, a 0.5 mL suspension of immature oocytes or maturing oocytes (15 min after the start of 1.0×10^{-6} 1-methyladenine treatment) was incubated at 20 °C for 30 min in ASW containing 200 μCi of [^{32}P]orthophosphate. After washing several times in ASW and twice in 1 M glucose, the oocytes were lysed by repeated freeze−thaw sequences in buffer B. After centrifugation at 16000*g* for 5 min, the supernatant was obtained. The samples were analyzed by polyacrylamide gel electrophoresis (PAGE) directly or after separation by immunoprecipitation as described below.

Polyacrylamide Gel Electrophoresis and Immunoblotting. SDS−PAGE was carried out according to the method described by Laemmli (*10*). For the separation of proteolytic digests of NAAPs, tricine-SDS−PAGE was carried out according to the method described by Schägger and Jagow (*11*). Two-dimensional gel electrophoresis was carried out according to the method described by O'Farrell et al. (*12*). Proteins on the gel were located by 0.25% Coomassie brilliant blue staining. For the detection of ^{32}P- or ^{35}S-labeled peptides, gels were dried and exposed to X-ray film (Kodak X-Omat AR) and intensifying screens. Densitomeric scans of autoradiograms were carried out using a bioimaging analyzer BAS-2000 (Fiji Film).

Immunostaining for NAAPs was performed using antisera generated in Balb/c mice, which had been immunized with purified NAAPs (HA grade) or a carboxyl-terminal sequence-specific antibody C raised in a rabbit. Western blot analysis was carried out as described by Towbin et al. (*13*) using PVDF membranes (Millipore) and horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G (Bio-Rad). Detection was performed using 3,3′-diaminobenzidine and H_2O_2.

Immunoprecipitation. For immunoprecipitation experiments, extracts of immature or maturing oocytes labeled in vivo with [^{32}P]orthophosphate or [^{35}S]methionine were precleared with Protein A-Sepharose CL-4B (Pharmacia Biotech) prior to incubation with anti-NAAP polyclonal antisera, preimmune sera, or antibody C in buffer IP, which contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, and 0.1% SDS, for 1 h at 4 °C. Protein A-Sepharose beads were added to each extract. After a 1 h incubation at 4 °C, each immunoprecipitate was washed four times with buffer IP, and once with 20 mM Tris-HCl (pH 7.5) and 0.5 M NaCl. The mixture was centrifuged at 500*g*

for 1 min, and the recovered beads were boiled in Laemmli's SDS sample buffer from which dithiothreitol was omitted and centrifuged to remove the beads, and the supernatant was subjected to SDS−PAGE under nonreducing conditions.

Nucleic Acid Binding Assay. For affinity binding with dsRNA, dsDNA, or single-stranded (ss) DNA, samples (500 ng of BSA-equivalent) in 100 μL of buffer B were incubated at 4 °C for 1 h with 30 μL of agarose-poly(I):poly(C) beads (Pharmacia Biotech), dsDNA−cellulose beads (Pharmacia Biotech), or ssDNA−cellulose beads (Pharmacia Biotech), respectively. As a control, the same amount of the sample was incubated with the same volume of agarose beads (Pharmacia Biotech) and processed in the same manner. After thorough washing with 10 mM Tris-HCl (pH 7.8), 6 mM $MgCl_2$, 80 mM KCl, 2 mM dithiothreitol, 250 mM sucrose and 0.1 mM EDTA, the beads were boiled in an SDS sample buffer (*10*) and were sedimented by a brief centrifugation. The supernatants were collected and analyzed by SDS−PAGE.

Digestion of NAAPs with Cyanogen Bromide. NAAPs were digested with cyanogen bromide in 70% formic acid at 4 °C for 36 h, as described (*14*). The solvents were removed by evaporation in vacuo and the residue was dissolved in 8 M urea. After removing the precipitates by brief centrifugation and filtration through Ultra-free C3GV filter (Millipore), the solution was loaded onto a RP-HPLC (Hi-Pore RP-318, Bio-Rad) column (4.6 × 250 mm). Fragments were eluted at a flow rate of 1 mL/min using a linear gradient of 5 to 55% acetonitrile (1%/min) in 0.1% TFA. The separated UV (OD at 214 nm)-absorbing fractions were collected, dried in vacuo, and their amino acid composition, mass values, and amino-terminal sequences determined.

Peptide Sequence Analysis. Purified NAAPs 1 and 2 were separately digested at 37 °C for 22 h with *Achromobacter lyticus* lysyl endopeptidase (Wako Chemicals, Osaka) using enzyme/substrate = 1/100 (w/w) in 20 mM Tris-HCl buffer (pH 9.0) containing 8% acetonitrile and 2 M urea. The resulting digests were separated by RP-HPLC using a Hi-Pore RP-318 column (4.6 × 250 mm) with a linear acetonitrile gradient (5 to 60%) in water. The amino acid sequence of each proteolytic fragment was determined by automated Edman degradation in an Applied Biosystem 477A/120A sequenator. Fragments which were generated by cyanogen bromide digestion were subjected to proteolysis at 37 °C for 20 h in 50 mM ammonium bicarbonate buffer (pH 7.8) with endoproteinase Asp-N (Boehringer Mannheim) using an enzyme/substrate ratio of 1/200 (w/w). The resulting digests were separated by RP-HPLC using an Inertsil ODS (GL Science, Tokyo) column (4.6 × 250 mm) with a linear acetonitrile gradient (5 to 100%) in 0.1% TFA. The amino acid sequence of each proteolytic fragment was determined by automated Edman degradation reactions.

Phosphorylation Site Analyses. NAAPs (HA grade) ^{32}P-labeled in vitro (2 μg of BSA-equivalent) and that ^{32}P-labeled in vivo (from 15 000 oocytes or eggs) were separately dissolved in 400 μL of buffer IP. They were immunoprecipitated by adding antibody C (20 μg of BSA-equivalent) and Protein A-Sepharose beads (Pharmacia Biotech: gel vol. = 20 μL). After tumbling the suspensions for 1 h at 4 °C, the immune complexes were collected by brief centrifugation in a microfuge. The immunoprecipitate was washed three times by resuspension in buffer IP and twice in 20 mM Tris-HCl (pH 7.5) and 0.5 M NaCl. The immunopellet was resuspended in 100 μL of 70% formic acid and ^{32}P-labeled NAAPs were extracted for 2 min. The beads were removed by brief centrifugation and the supernatant was collected. The extract was treated with cyanogen bromide as described (*14*). Fragments produced were resolved by tricine-PAGE (*11*). The peptide markers were located by Coomassie staining and the radioactive bands by autoradiography. For phosphopeptide analysis, immunoprecipitates prepared in the same way were extracted with 70% formic acid. After removal of the solvents in the extract by evaporation in vacuo, the residue was dissolved in 10 mM NH_4HCO_3 (pH 7.8) and incubated with 1 μg of trypsin (Boehringer-Mannheim) at 37 °C for 12 h. After boiling for 5 min, the reaction mixture was further incubated with 1 μg of endoproteinase Glu-C (Promega) at 37 °C for 24 h. The reaction mixture was separated by thin-layer chromatography on a cellulose plate using a solvent system of *n*-butanol−acetic acid−pyridine−water (75:50:15:60, vol/vol). Phosphopeptides were visualized by autoradiography.

Phosphoamino acid analyses were carried out as described by Cooper et al. (*15*). The positions of authentic phosphoamino acids were visualized by ninhydrin staining.

Mass Spectrometry. MALDI-TOF-MS was performed using a Voyager Elite mass spectrometer (PE Biosystems) at the acceleration voltage of 20 kV. Positive-ion MALDI-TOF-MS/MS spectra were obtained with the same instrument. Experimental details of this procedure have been described previously (*16*).

Preparation of Starfish Ovary cDNA Libraries. A total DNA from λ ZAP cDNA library prepared from young *A. pectinifera* ovaries according to the method described by Tachibana et al. (*17*) was used for PCR with degenerated sense and antisense primers deduced from the amino acid sequences Glu-Gly-Glu-Arg-Asn-Val-Val-Glu and Asp-Gly-Asp-Asn-Val-Lys, respectively (*18*). A synthetic oligonucleotide containing the coding region of Glu-Ile-Glu-Thr-Glu-Asn-Phe was used as the ^{32}P-labeled probe for the screening of the library (*18*).

Northern Hybridization. Total RNA from a young *A. pectinifera* ovary was prepared and subjected to electrophoresis on a formaldehyde/agarose gel as described by Tachibana et al. (*17*). The resolved RNA was blotted onto Nylon membranes by capillary transfer and prehybridized at 42 °C overnight in a prehybridization buffer [5× SSC (0.75 M NaCl and 75 mM trisodium citrate (pH 7.0), 0.02% SDS, 50% formamide, and 0.1% *N*-lauroylsarcosine, 0.1 mg/mL herring sperm DNA, and 2% blocking reagent (Boehringer Mannheim)]. Hybridization and washing were carried out at 42 and 68 °C, respectively. The probe corresponds to a fragment of ca. 820 base-paired nucleotides obtained by double digestion of an NAAP2-encoding cDNA clone with *Eco*RI and *Sca*I, which had been labeled with digoxigenin by random priming using a digoxigenin DNA labeling and detection kit (Boehringer Mannheim). After hybridization, the membrane was washed and analyzed for digoxigenin according to the manufacturer's recommended protocol.

RESULTS

Purification of NAAPs. It has been shown that several proteins in starfish oocytes are phosphorylated when matur-

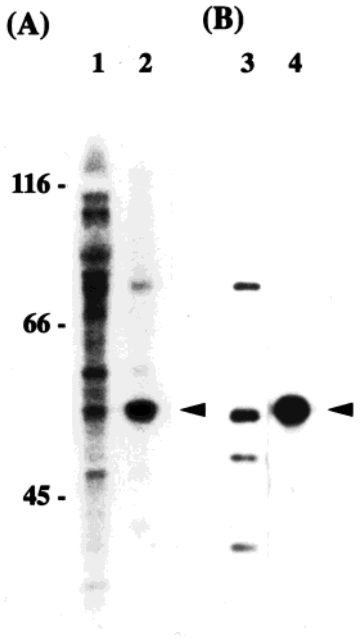

FIGURE 1: Phosphorylation of NAAPs 1 and 2 in vivo and in vitro. (A) Maturing oocytes incubated at 20 °C with [^{32}P]H_3PO_4 for 30 min were extracted in buffer A and the extract was incubated with agarose-poly(I)·poly(C) beads. The fraction which bound to the beads was analyzed by SDS−PAGE, followed by autoradiography. Lane 1, egg extract; lane 2, agarose-poly(I)·poly(C)-bound fraction of the egg extract. (B) The extract of immature oocytes was incubated at 20 °C for 30 min in the presence of [γ-^{32}P]ATP in buffer B, then with agarose-poly(I)·poly(C) beads. The fraction which bound to the beads was analyzed by SDS−PAGE and autoradiography. Lane 3, unseparated reaction mixture; lane 4, agarose-poly(I)·poly(C)-bound fraction. Arrowheads indicate the position of NAAPs which were purified to homogeneity in this study. M_rs $\times 10^{-3}$ of size marker proteins are indicated on the left.

ing oocytes are incubated with [^{32}P]orthophosphate whereas this is not the case for immature, G_2-arrested oocytes when they were incubated in a similar manner (*18*). Since the cdc2 kinase is active at the first meiotic prometaphase but inactive at the first meiotic G_2 phase, these proteins represent possible candidates for substrates of p34^{cdc2}/cyclin B. To obtain ^{32}P-labeled, dsRNA-binding proteins in an extract of eggs which had been phosphorylated in vivo, the extract was incubated with agarose-poly(I)·poly(C) beads, and the fraction which bound to these beads was subjected to SDS−PAGE, followed by autoradiography. The affinity-purified fraction gave a single band at M_r 50 kDa on a gel (Figure 1A) as described previously (*18*).

To purify the 50 kDa protein, we first attempted to utilize maturing oocytes as the starting material. However, we were unable to obtain consistent elution profile of DEAE-cellulose chromatography, possibly due to variable amounts of phosphorylated and hypophosphorylated forms of the protein in extracts from different batches of maturing oocytes. Although the rate of in vivo phosphorylation in immature oocytes is quite low, a substantial level of phosphorylation was observed when an oocyte extract was incubated with [γ-^{32}P]-ATP in buffer B (Figure 1B). The optimum pH of the in vitro phosphorylation reaction was 7.8, and the phosphorylation required the presence of Mg^{2+} or Ca^{2+} ions. The presence of calmodulin, cyclic AMP or cyclic GMP was inhibitory, and the presence of L-α-phosphatidyl-L-serine along with Ca^{2+} ion did not significantly affect kinase activity (data not shown). To obtain dsRNA-binding proteins, which had been phosphorylated in vitro in the extract, it was incubated with agarose-poly(I)·poly(C) beads, and the poly-(I)·poly(C)-binding proteins were pulled down by centrifugation. SDS−PAGE of the agarose-poly(I)·poly(C)-bound proteins followed by autoradiography revealed the presence of a ^{32}P-labeled poly(I)·poly(C)-binding protein which migrated at M_r 50 kDa and which appeared to be identical to the ^{32}P-labeled poly(I)·poly(C)-binding protein phosphorylated in vivo (Figure 1, lanes 2 and 4). The ability to bind to dsRNA and susceptibility to a protein kinase present in the extract of immature oocytes formed the basis of detecting the possible candidates for substrates of p34^{cdc2}/cyclin B during the course of their purification.

An oocyte extract was subjected to DEAE-cellulose chromatography, and each fraction was incubated with [γ-^{32}P]ATP in buffer B. Fractions, which contained ^{32}P-labeled protein with M_r 50 kDa (fractions 4−7, Figure 2A), were combined and then affinity-purified using agarose-poly-(I)·poly(C) beads. When in vitro phosphorylation was carried out on a suspension of agarose-poly(I)·poly(C)-beads to which had been adsorbed the 50-kDa protein in fractions 4−7 of DEAE-cellulose chromatography, a single radioactive spot at M_r 50 kDa and pI 3.6 was detected by two-dimensional gel electrophoresis as revealed by Coomassie brilliant blue staining and autoradiography (Figure 2B). Therefore, the protein kinase which phosphorylates 50 kDa protein is associated with this substrate protein. The amount of the kinase was too low to permit its detection by Coomassie staining. The kinase can be dissociated from the substrate protein by HA chromatography: the purified 50 kDa protein (HA grade) was not phosphorylated by incubation with [γ-^{32}P]ATP in buffer B unless an egg extract was added to the reaction mixture (Figure 2C). Similarly, an extract of immature oocytes supported phosphorylation of NAAPs (HA grade) in buffer B containing [γ-^{32}P]ATP (data not shown). Phosphoamino acid analysis of the phosphorylated 50 kDa protein showed that a Ser residue(s) were phosphorylated (data not shown). Since a severe inhibition of phosphorylation was observed when the reaction was carried out in the presence of heparin sulfate, a specific inhibitor of casein kinase II, it is possible that this protein-serine/threonine kinase is responsible for the observed phosphorylation.

Although the apparent homogeneity of the purified 50 kDa protein was confirmed by one- and two-dimensional PAGE systems (Figure 2B), the protein (HA grade) was further purified by HPLC using a RP C4 column. In this system, two major peaks with UV (OD at 230 nm)-absorbance were observed narrowly separated from one another (Figure 3A). We tentatively designated the protein which eluted first as NAAP1 and that eluting later as NAAP2.

Determination of the Chemical Structure of NAAPs 1 and 2. To determine the difference in chemical structure between NAAP1 and NAAP2, they were individually digested with cyanogen bromide, to cleave the carboxyl-terminal side of the Met residues and the digests were then separated by RP-HPLC using a Hi-Pore RP-318 column. Five major UV (OD at 214 nm)-absorbing fragments were obtained from each NAAP digest (Figure 3B) and each was subjected to MALDI-TOF-MS, which gave the mass values shown in Table 1.

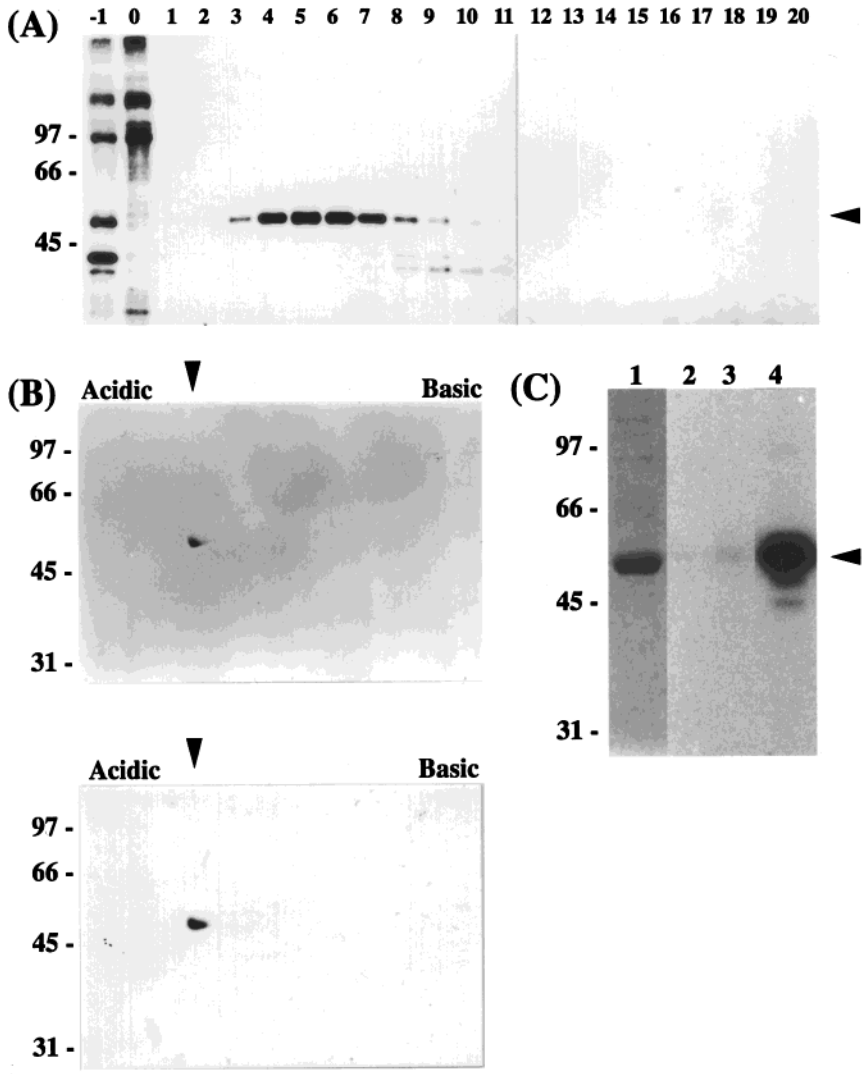

FIGURE 2: Phosphorylation of partially purified NAAPs 1 and 2 in vitro. (A) The extract of immature oocytes was subjected to DEAE-cellulose column chromatography. Aliquots of separated fractions were individually incubated at 20 °C for 10 min in the presence of [γ-^{32}P]ATP in buffer B and the reaction products were analyzed by SDS−PAGE and autoradiography. Lane −1, unseparated oocyte extract; lane 0, break through fraction; lanes 1−20, fractions 1−20, respectively. An arrowhead indicates the position of NAAPs which have been purified to homogeneity in this study. M_rs $\times$ 10^{-3} of size marker proteins are indicated on the left. (B) NAAP-containing fractions from DEAE-cellulose chromatography were incubated with agarose-poly(I)·poly(C) beads. The fraction which bound to the beads was incubated at 20 °C for 10 min in the presence of [γ-^{32}P]ATP in buffer B and the reaction products were analyzed by two-dimensional gel electrophoresis, followed by Coomassie brilliant blue staining (upper panel) and autoradiography (lower panel). M_rs $\times$ 10^{-3} of size marker proteins are indicated on the left. Arrowheads indicate the position of the pI marker protein, *Aspergillus niger* amyloglucosidase (pI 3.6). (C) NAAP-containing fractions of DEAE-cellulose chromatography were further purified by hydroxylapatite chromatography. Fractions which were homogeneous in NAAPs (HA grade) were combined and incubated at 20 °C for 10 min with [γ-^{32}P]ATP in buffer B in the presence or absence of an egg extract (4 μg of BSA-equivalent), and the reaction products were analyzed by SDS−PAGE followed by Coomassie brilliant blue staining (lane 1) and autoradiography (lanes 2−4). Lanes 1 and 2, NAAPs (HA grade); lane 3, egg extract; lane 4, NAAPs (HA grade) plus egg extract. An arrowhead indicates the position of NAAPs. M_rs $\times$ 10^{-3} of size marker proteins are indicated on the left.

CB-I-1 and CB-I-2 were individually digested with Asp-N protease, and the resulting fragments were separated by RP-HPLC using an Inertsil-ODS column (Figure 4A). The major difference between these chromatograms was found in the abundance of two UV (OD at 214 nm)-absorbing peaks (indicated by D3 and D4 in Figure 4A). Amino acid sequences of these fragments were determined by the automated Edman degradation reaction. The sequence of the peptide in peak D3 (Figure 4A) was Asp^{1}-Gly-Ile-Gln-His-Phe-Leu-Phe-Leu-Lys-Gln-Ala-Val-Leu-Gly-Ala-Asn^{17}-Ala-Lys-Glu-Gly-Glu-Arg-Asn-Val-Val-Glu-Ile-Glu-Thr-Glu-Asn-Phe^{33} (Figure 4B). The sequence of the peptide in peak D4 (Figure 4A) was identical to the peptide in peak D3 except that the residue 17 of the former was Thr, rather than Asn (Figure 4B). This single amino acid substitution explains the difference of the molecular ion of CB-I-1 and that of CB-I-2. The former is 13 mass units larger than the latter (Table 1), which is consistent with the above findings.

A comparison of the observed molecular ions for CB-II, -III, -IV, and -V derived from NAAP1 and those derived from NAAP2 (Table 1) indicated that no obvious differences in amino acid sequences between the NAAP1-derived fragments and the corresponding counterparts derived from NAAP2 are present. Furthermore, RP-HPLC of CB fragments produced by digestion with Agr-C, V8 protease, or *A. lyticus* lysyl endopeptidase indicated no noticeable differences in their chromatograms between each pair of CB fragments (data not shown). It thus appears that the sequence

Table 1: MALDI Mass Values of Peptides Produced from the Digestion of NAAPs 1 and 2 with Cyanogen Bromide

peak in RP-HPLC (Figure 3B)	CNBr fragment (amino acid residues)	theoretical molecular weight[a]		observed molecular weight	
		homoserine	homoserine lactone	NAAP1 (*m*/*z*)	NAAP2 (*m*/*z*)
C1	CB-III (170−195)	3055	3036.98	3035.53 3053.49	3038.25 3056.25
C2	CB-IV (199−262)	7417.93	7399.91	7200−7500	7200−7500
C3	CB-II (116−169)	6006.33	5988.31	5986.62 6007.5 6086.34	5987.7 6011.48 6089.38
C4	CB-V (263−345)	9358.9		9358.67	9359.33
C5−1	CB-I-1 (1−115)	12 654.04	12 636.02	12 698	
C5−2	CB-I-2 (1−115)	12 654.04	12 636.02		12 683.9

*The values are based on the putative amino acid sequence derived from the sequence of NAAP2 cDNA.

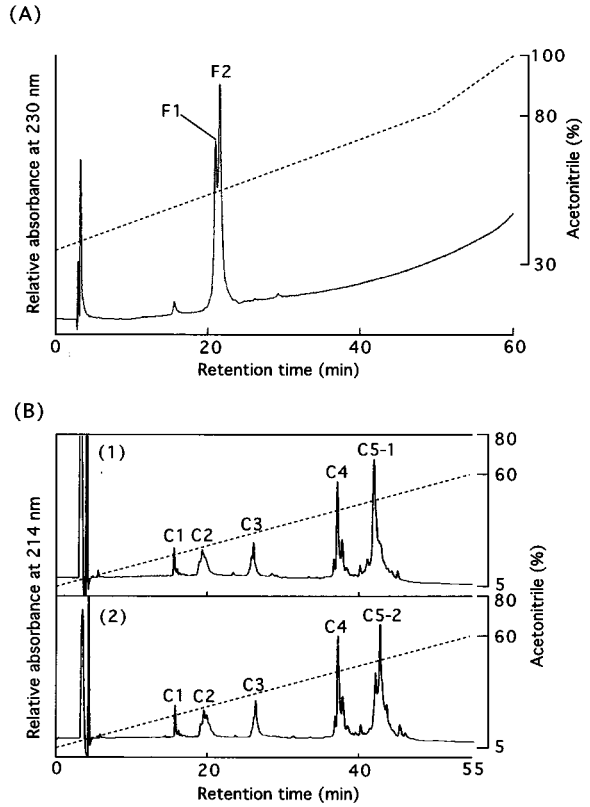

FIGURE 3: Separation of NAAPs 1 and 2, and their cyanogen bromide digests by RP-HPLC. (A) NAAPs (HA grade) were purified by RP-HPLC using a μBondasphere C4−300 Å column (3.9 × 150 mm). The elution was carried out with an acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min. The absorbance at 230 nm was monitored and shown by a solid line. The concentration of acetonitrile is shown by a dotted line. Peaks F1 and F2 correspond to NAAP1 and NAAP2, respectively. (B) Separation of peptides obtained by cyanogen bromide digestion of NAAP1 (1) and NAAP2 (2) by RP-HPLC using a Hipore RP-318 column (4.6 × 250 mm). The elution was carried out with an acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. The relative absorbance at 214 nm is shown by solid lines. The concentrations of acetonitrile are shown by dotted lines.

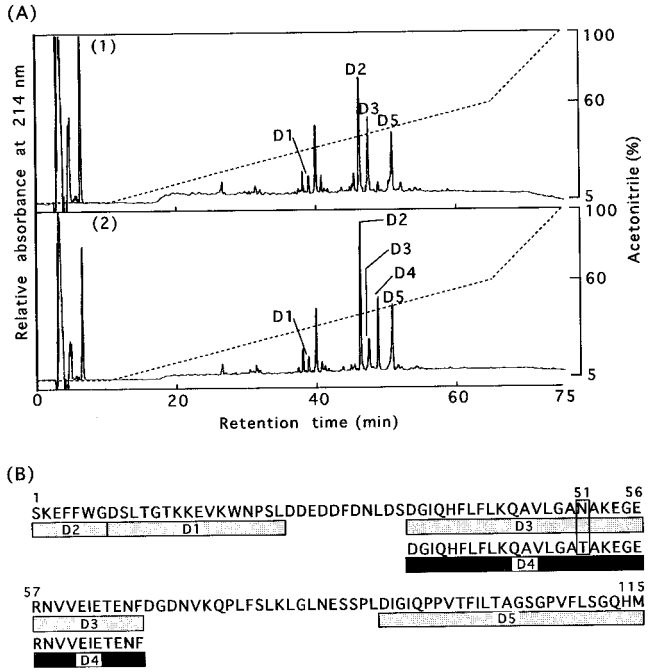

FIGURE 4: Chromatographic separation of peptides produced from Asp-N digestion of CB-I-1 and CB-I-2, which were derived from NAAP1 and NAAP2, respectively. (A) Digests of CB-I-1 (1) and CB-I-2 (2) were separately subjected to RP-HPLC using an Inertsil ODS column (4.6 × 250 mm). The elution was carried out with an acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. The relative absorbance at 214 nm is shown by solid lines. The concentrations of acetonitrile are shown by dotted lines. (B) Amino acid sequences of the peptides in peaks D1, D2, D3, D4, and D5 of RP-HPLC (A).

difference between NAAP1 and NAAP2 could have been derived from only one amino acid substitution described above.

NAAP1 and NAAP2 were individually digested with *A. lyticus* lysyl endopeptidase, the fragments were separated by RP-HPLC, and their sequences determined by conventional microsequencing. A peptide, with a sequence of Glu-Gly-Glu-Arg-Asn-Val-Val-Glu-Ile-Glu-Thr-Glu-Asn-Phe-Asp-Gly-Gly-Asp-Asn-Val-Lys was obtained from each protein, together with eight other peptides (Figure 5). The above sequence was used to design degenerate oligonucleotide primers and a hybridization probe, which is internal to the PCR primers, and these were utilized to amplify and sequence the PCR product that codes for the portion of NAAPs. Total RNA of a young *A. pectinifera* ovary was used as a template for reverse transcription-PCR. The ^{32}P-end-labeled oligodeoxyribonucleotide which codes for the peptide was used to screen a λ ZAP cDNA library made

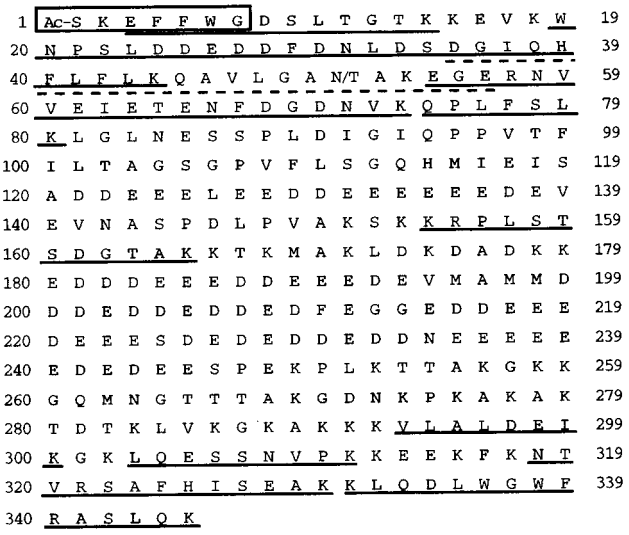

FIGURE 5: Amino acid sequences (single letter code) of NAAPs 1 and 2. The peptide sequence identified by MS/MS analysis is indicated by boxes and the sequences obtained by amino acid sequencing are underlined by solid or dotted lines. The other parts of the sequences were deduced from those of the NAAP2 cDNA insert of pBS-NAAP (DDBJ accession no. AB037176). The amino acid residues at position 51 of NAAPs 1 and 2 are Asn and Thr, respectively.

from poly(A)$^+$RNA which was isolated from young *A. pectinifera* ovaries. These screens identified several positive clones, which were converted into the pBluescript SK(−) phagemid and sequenced. The amino acid sequence deduced from the nucleotide sequence of one positive clone, referred to as pBS-NAAP, is shown in Figure 5. The clone consists of 2106 base pairs and contains a single open reading frame which encodes 346 amino acids including a putative initiating Met residue with a predicted M_r value of 38 992 and a pI of 3.5. The putative amino acid sequence contains the sequence of the peptide in peak D4 of RP-HPLC (Figure 4A), which was derived from the Asp-N digestion of NAAP2, but the sequence of the peptide in peak D3 (Figure 4A), which was derived from Asp-N digestion of NAAP1, is absent.

Another clone, which was designated as clone 6-1, was found to encode NAAP1 which was referred to as ANO39 (*18*).

NAAPs 1 and 2 were individually subjected to microsequencing, but no sequence data were obtained, indicating that their amino-termini are blocked. The peptide in peak D2 of RP-HPLC (Figure 4A) obtained from both CB-I-1 and CB-I-2 was shown to contain a blocked amino-terminal residue and, therefore, was analyzed by MALDI-TOF-MS/MS (Figure 6). The structure of the peptide in peak D2 (Figure 4A) was determined to be Ac-Ser^1-Lys-Glu-Phe-Phe-Trp-Gly^7. This suggests, therefore, that the initiation Met residue was removed and that N-acetylation took place on the second Ser residue. It follows therefore that both NAAP1 and NAAP2 contain 345 amino acid residues (Figure 5). No Cys residues are present in these NAAPs, indicating that intrachain or interchain disulfide bonds are not involved in their folding.

The most conspicuous feature of the NAAPs is the presence of clusters of negatively charged amino acid residues at the central region and the carboxyl-terminal basic domain. The M_r value predicted from the cDNA sequence

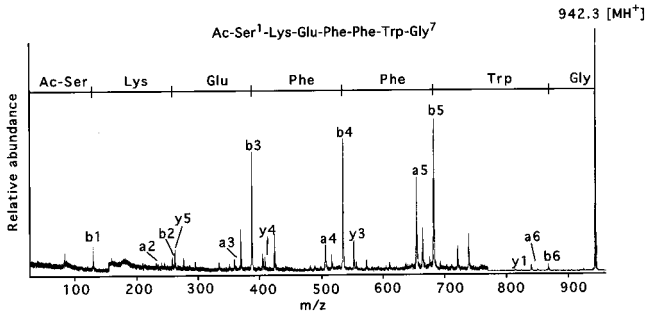

FIGURE 6: Positive-ion-MALDI-TOF-MS/MS analysis of the peptide in peak D2 of RP-HPLC (Figure 4A) obtained by digestion of NAAP1 or NAAP2 with proteinase Asp-N. The nomenclature of sequence ions for the peptide is as described elsewhere (*43*).

is smaller than the M_r value of 50 000, estimated from SDS−PAGE (Figure 2B). Such obvious deviations in M_r estimations and predicted M_r values have also been reported for several other proteins which contain clusters of negatively charged amino acid residues and appear to be due to lesser SDS binding and, hence, an anomalously low electrophoretic mobility (*19*−*22*).

Molecular and Biochemical Characterization of NAAPs. Northern blot hybridization on total RNA from a young *A. pectinifera* ovary which contained oocytes arrested at the meiotic G_2 phase yielded a strong signal at ∼2.0 kb (Figure 7A). The protein synthesized in vivo by the incubation of immature oocytes with [^{35}S]methionine was efficiently immunoprecipitated from their lysate with a carboxyl-terminal sequence-specific antibody C, which was raised in a rabbit by giving several injections of a keyhole-limpet hemocyanin-coupled carboxyl-terminal peptide of NAAPs, Lys-Leu-Gln-Asp-Leu-Trp-Gly-Trp-Phe-Asn-Ala-Ser-Leu-Gln-Lys, with an additional amino-terminal Cys residue (Figure 7B, lane 1). On the other hand, no radioactive proteins were immunoprecipitated from a lysate of maturing oocytes, which were incubated with [^{35}S]methionine (Figure 7B, lane 2). Immunoblot analysis using antibody C as the probe indicated that the amount of NAAPs present in maturing oocytes is nearly the same as that which is present in the G_2-arrested oocytes (Figure 7C, lanes 1 and 2). These results suggest that genes which code for NAAPs are transcribed and the produced mRNAs are translated in the oocyte at the G_2 stage and that the transcripts are lost or not translated during oocyte maturation. To address the issue of whether NAAPs are present in the embryo, immunoblot analysis was performed using antibody C as the probe on the electrophoresed proteins of oocytes and embryos at different developmental stages. The results shown in Figure 7D clearly demonstrate that the signal of NAAPs on the immunoblot from extracts of oocytes and embryos at the morula through mid-gastrula stage was nearly the same although the band of NAAPs shifted from a high- to a low-mobility from between oocytes and the 128-cell embryos, possibly due to an unknown phosphorylation on other amino acid residues than Ser^{144} (bee below) which has not been pursued in this study. To determine the subcellular localization of NAAPs, mid-blastulae and mid-gastrulae were separated into cytosolic and nuclear fractions, and then equal amounts of proteins from each fraction were electrophoresed and immunoblotted with antibody C. Immunoblots shown in Figure 7E demonstrate that NAAPs are mostly located in

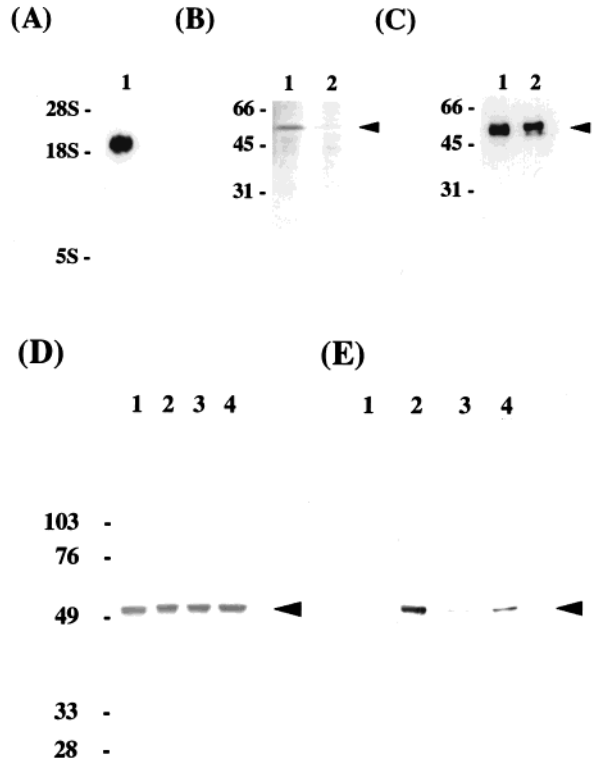

FIGURE 7: Translation of NAAP mRNA in oocytes after resumption of meiotic arrest. (A) Northern blot of total RNAs prepared from an unripe ovary filled with G_2-arrested oocytes was hybridized in 50% formamide at 42 °C with a digoxigenin-labeled DNA probe obtained from the 820 base-paired nucleotides obtained by digestion of the NAAP2-encoding cDNA clone with *Eco*RI and *Sca*I. On lane 1 was loaded 20 μg of total RNA. The positions of ribosomal RNAs are indicated on the left. (B) Synthesis of NAAPs in vivo before and after initiation of meiotic resumption. Cultures of immature oocytes and of maturing oocytes were labeled with [^{35}S]-methionine. NAAPs were collected by immunoprecipitation using antibody C from lysates of immature oocytes (lane 1) and of maturing oocytes (lane 2). These were subjected to SDS−PAGE followed by autoradiography. An arrowhead indicates the position of NAAPs and M_rs $\times$ 10^{-3} of size marker proteins are indicated on the left. (C) Immunoprecipitation of NAAPs with antibody C from lysates of immature oocytes (lane 1) and of maturing oocytes (lane 2). The proteins were electrophoresed on SDS−PAGE, transferred to PVDF membrane, and probed with antibody C. An arrowhead indicates the position of NAAPs and M_rs $\times$ 10^{-3} of size marker proteins are indicated on the left. (D) Western blot analysis of NAAPs during embryogenesis. Oocytes or embryos at different developmental stages were extracted with buffer A. Proteins in each extract (150 μg of BSA-equivalent) were separated by SDS−PAGE, transferred to PVDF membrane, and probed antibody C. Lane 1, oocytes; lane 2, 128-cell-stage embryos (6-h-old embryos); lane 3, mid-blastulae (12-h-old embryos); lane 4, mid-gastrulae (24-h-old embryos). An arrowhead indicates the position of NAAPs and M_rs $\times$ 10^{-3} of size marker proteins are indicated on the left. (E) Western blot analysis of NAAPs in the subcellular fractions of embryos after blastulation. Mid-blastulae (12-h-old-embryos) and mid-gastrulae (24-h-old embryos) were fractionated into nuclear and cytosolic fractions. Proteins in each fraction (50 μg of BSA-equivalent) were separated by SDS−PAGE, transferred to PVDF membrane, and probed with antibody C. Lane 1, cytosolic fraction of mid-blastulae; lane 2, nuclear fraction of mid-blastulae; lane 3, cytosolic fraction of mid-gastrulae; lane 4, nuclear fraction of mid-gastrulae. An arrowhead indicates the position of NAAPs.

the nucleus. These results indicate that NAAPs are maternal proteins which are produced by translation of mRNA in the immature oocyte and distribute to the nucleus of early embryos after passing through a series of oogenetic and developmental stages, i.e., oocyte maturation, fertilization, early embryonic cleavages and embryonic morphogenesis.

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p62      373  KPKKEEKFKNFVKSKFHLSEGKKIQELWGWYKSTQLTAK  411
              |||||||||.|.| ||.||.||.|.||||....
NAAP2    309  VPKKEEKFKNYVRSAFHISEAKKLQDLWGWFRASLQK    345
              || |.|| |||.. |..... .|||| | .. |
B23.1    261  LPKVEAKFINYVKNCFRMTDQEAIQDLWQWRKS-L      294
              |||||.|| |||||||| ..|..|||||.||.| |
NO38     255  LPKVEVKFANYVKNCFRTDSQKVIQDLWKWRQS-LKDGK  292
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FIGURE 8: Amino acid sequence comparison of the carboxyl-terminal region of NAAPs with that of sea urchin p62 (*23*), *Xenopus laevis* NO38 (*19*), and human B23.1 (*24*). The deduced amino acid sequence of NAAP2 was aligned with p62, NO38, and B23.1 using a FASTA comparison program. The software indicates identical residues with vertical lines between given pairs of residues, while conservative substitutions are indicated by a single dot.

NAAPs showed some degree of similarity (overall 41%) to p62 of the sea urchin (*Strongylocentrotus purpuratus*) eggs and embryos, a substrate of Ca^{2+}/calmodulin-dependent protein kinase which is associated with p62 (*23*). NAAPs showed 29.5 and 32.7% overall similarity to the nucleolar protein NO38 of the *Xenopus laevis* oocyte (*19*) and human nucleolar protein B23.1 (*24*), respectively. This similarity is due to the extensive stretches of Glu and Asp residues that occur in these proteins. The significant sequence homology is confined to a stretch of amino acids at the carboxyl-terminal regions (Figure 8) but little sequence similarity is observed in the remainder of these proteins. B23.1 is capable of binding to DNA and a 5 kDa carboxyl terminal fragment of B23.1 retains nearly all of the DNA-binding activity of the parent protein (*25*).

To determine whether NAAPs associate with DNA, purified NAAPs (HA grade) were applied to cellulose beads which bound native or denatured DNA. Under the experimental conditions described in the Materials and Methods, NAAPs bind to both dsDNA−cellulose and ssDNA−cellulose at a comparable level as was observed for dsRNA-bound agarose beads (Figure 9, lanes 2−4). No detectable preference of NAAPs for a specific type of nucleic acid was observed. As a control, Sepharose beads, instead of nucleic acid bound beads, were used and processed in a similar manner. NAAPs failed to bind to the beads (Figure 9, lane 5), indicating that the binding of NAAPs to the nucleic acid−cellulose beads is due to nucleic acid binding and not to nonspecific interaction with the cellulose matrix.

Phosphorylation of NAAPs in Vivo. To determine whether mitotic phosphorylation of NAAPs occurred in vivo, NAAPs were immunoprecipitated from G_2-arrested or maturing oocytes after labeling with [^{32}P]orthophosphate (Figure 10A). Whereas NAAPs, which were obtained from immature oocytes, were not radioactive (Figure 10A, lane 1), those from oocytes which progressed to the first meiotic prometaphase to metaphase (15−45 min after the initiation of 1-methyladenine treatment) contained radioactivity (Figure 10A, lane 2). Phosphoamino acid analysis of NAAPs that were isolated by immunoprecipitation from ^{32}P-labeled maturing oocytes indicated that the proteins had been phosphorylated exclusively on a Ser residue(s) (*18*). An aliquot of the immunopellet was subjected to proteolysis with cyanogen bromide. Separation of the digest by tricine-SDS−PAGE (*11*) afforded ^{32}P-labeled CB-II as the sole radioactive fragment as revealed by autoradiography (Figure 10B, lane 1). An aliquot of the immunopellet was next digested exhaustively with trypsin and proteinase Glu-C. Thin-layer chro-

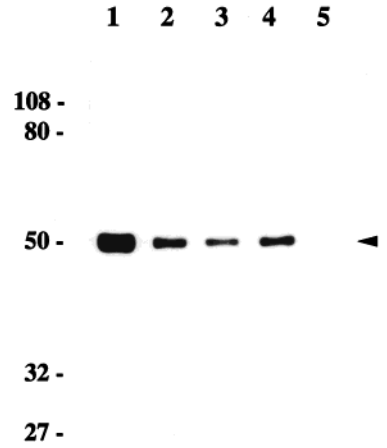

FIGURE 9: Nucleic acid binding capacity of NAAPs. Five hundred nanograms of purified NAAPs was incubated in a buffer containing 30 μL of nucleic acid-bound or unbound beads. The fractions which bound to the beads were analyzed by SDS−polyacrylamide gel electrophoresis and subsequent Western blot staining using an anti-NAAP antibody. Lane 1, untreated NAAPs; lane 2, agarose-poly-(I):poly(C)-bound fraction; lane 3, dsDNA−cellulose-bound fraction; lane 4, ssDNA−cellulose-bound fraction; and lane 5, Sepharose 4B-bound fraction. An arrowhead indicates the position of NAAPs and M_rs $\times$ 10^{-3} of size marker proteins are indicated on the left.

matography of the digest afforded a single radioactive spot the mobility of which was the same as that of the synthetic phosphopeptide Val[141]-Gln-Ala-phosphoSer-Pro-Asp-Leu-Pro-Val-Ala-Lys[151] (*18*), but which was different from the synthetic phosphopeptide Asp[146]-Leu-Pro-Val-Ala-Lys-phosphoSer-Lys-Lys-Arg-Pro-Leu-Ser-Thr-Ser[160] (Figure 10C, lane 1). These results showed that Ser[144] is the amino acid residue that is phosphorylated during oocyte maturation (*18*).

Phosphorylation of NAAPs by Purified Starfish p34cdc2/cyclin B. To determine possible relationships between the mitotic phosphorylation of the NAAPs and their ability to serve as in vitro substrates for cdc2 kinase, we carried out a comparative phosphopeptide analysis. The kinase p34^{cdc2}/cyclin B which was highly purified from *A. pectinifera* eggs readily phosphorylated NAAPs (HA grade) (Figure 10A, lane 3). Phosphoamino acid analysis revealed that the cdc2 kinase phosphorylated only a Ser residue(s) (*18*). Tricine-SDS−PAGE of fragments generated by cyanogen bromide digestion of NAAPs which had been phosphorylated in vitro revealed that CB-II was the only phosphorylated fragment (Figure 10B, lane 2). Double digestion of NAAPs (HA grade) phosphorylated in vitro with trypsin and Glu-C, followed by thin-layer chromatography, gave a single spot on the plate as revealed by autoradiography. The relative mobility of the spot was the same as that of the phosphopeptide derived from the NAAPs, which had been labeled in vivo (Figure 10C, lane 2). These results demonstrate that the NAAPs were phosphorylated at the Ser[144] residue by p34^{cdc2}/cyclin B, which is activated after reinitiation of the meiotic process (*18*).

The kinase p34^{cdc2}/cyclin B is thought to require the consensus sequence (Ser/Thr)-Pro-X-Z, where X is any amino acid and Z is a basic amino acid (*26*). Although such evolutionarily conserved motif (Ser[246]-Pro-Glu-Lys) exists in the sequences of NAAPs and CB-IV, mitotic phosphorylation of NAAPs by p34^{cdc2}/cyclin B occurs on a different site (Figure 10B,C).

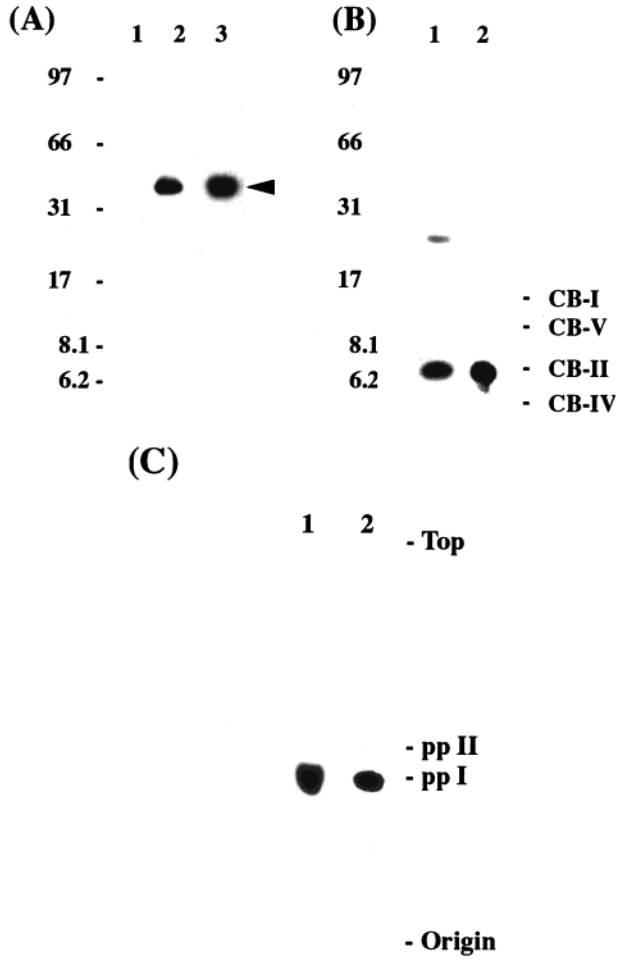

FIGURE 10: Analysis of the phosphoacceptor site in NAAPs which were phosphorylated in vivo and in vitro by p34^{cdc2}/cyclin B. (A) Immature and maturing oocytes were labeled with [^{32}P]H_3PO_4 and extracted with buffer A. NAAPs were collected by immunoprecipitation using antibody C from lysate of immature oocytes (lane 1) and of maturing oocytes (lane 2). NAAPs (HA grade) which were phosphorylated in vitro with [γ-^{32}P]ATP by p34^{cdc2}/cyclin B were collected by immunoprecipitation using antibody C (lane 3). They were subjected to SDS−PAGE followed by autoradiography. An arrowhead indicates the position of NAAPs and M_rs $\times$ 10^{-3} of size marker proteins are indicated on the left. (B) An aliquot of the immunoprecipitate from the ^{32}P-labeled maturing oocytes or from in vitro labeled NAAPs (HA grade) was digested with cyanogen bromide and the digests were separated by tricine-SDS−PAGE, followed by autoradiography. Lane 1, a digest of in vivo labeled NAAPs; lane 2, a digest of in vitro labeled NAAPs. M_rs $\times$ 10^{-3} of size marker peptides are indicated on the left and the positions of CB-I, CB-II, CB-IV, and CB-V on the right. (C) An aliquot of the immunoprecipitate from ^{32}P-labeled maturing oocytes or from in vitro labeled NAAPs (HA grade) was digested with trypsin and proteinase Glu-C. The digests were separated by thin-layer chromatography, followed by autoradiography. Lane 1, a digest of in vivo labeled NAAPs; lane 2, a digest of in vitro labeled NAAPs. The positions of marker peptides, pp I (Val[141]-Gln-Ala-phosphoSer-Pro-Asp-Leu-Pro-Val-Ala-Lys[151]) and pp II (Asp[146]-Leu-Pro-Val-Ala-Lys-phosphoSer-Lys-Lys-Arg-Pro-Leu-Ser-Thr-Ser) are indicated on the right.

To understand the factors that determine the target specificity for the mitotic cdc2 kinase, we tested the ability of CB fragments to serve as in vitro substrates for p34^{cdc2}/cyclin B. Phosphorylation of peptides was analyzed by tricine-PAGE, followed by autoradiography. As shown in

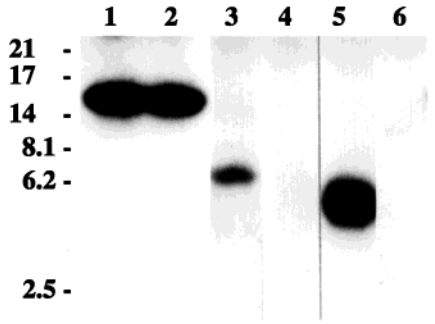

FIGURE 11: Phosphorylation in vitro by p34^{cdc2}/cyclin B of fragments derived from digestion of NAAPs (HA grade) with cyanogen bromide. Fragments CB-I-1 (lane 1), CB-I-2 (lane 2), CB-II (lane 3), CB-III (lane 4), CB-IV (lane 5), and CB-V (lane 6) (200 pmol each) were individually phosphorylated in the presence of [γ-^{32}P]ATP by p34^{cdc2}/cyclin B. The reaction mixtures were separated by tricine-SDS−PAGE, followed by autoradiography. M_rs $\times 10^{-3}$ of size marker peptides are indicated on the left.

Figure 11 (lane 3), CB-II which contains the Ser^{144} residue in NAAPs served as a phosphoacceptor site. However, CB-IV which contains the sequence Ser^{246}-Pro-Glu-Lys (Figure 11, lane 5) and CB-I which contains Ser^{87}-Pro-Leu-Asp (Figure 11, lanes 1 and 2) were shown to be phosphorylated to a much greater extent than CB-II. These results suggest that the tertiary structure of NAAPs affects the target specificity of the cdc2 kinase.

DISCUSSION

The p34^{cdc2}/cyclin B complex plays a pivotal role in the regulation of the eukaryotic cell cycle (*26*, *27*). It is clearly important, therefore, to define the physiological substrate range of this kinase. This report describes two related nuclear proteins of starfish oocytes designated as NAAPs 1 and 2, as in vivo substrates for the cdc2 kinase. Specifically, the data herein show that NAAPs are phosphorylated during meiotic maturation in vivo, and the two proteins are readily and specifically phosphorylated by highly purified starfish p34^{cdc2}/cyclin B in vitro. Moreover, we demonstrated, via proteolytic phosphopeptide mapping, that the cdc2 kinase phosphorylates the same site in vitro that is phosphorylated during mitosis in vivo (*18*). The most straightforward interpretation of these results is that the nuclear proteins NAAPs are direct in vivo substrates of mitotically activated p34^{cdc2}/cyclin B.

Work on p34^{cdc2}/cyclin B has revealed that the kinase requires a (Ser/Thr)-Pro-X-Z motif (where X is any amino acid and Z is a basic amino acid) as the consensus sequence for phosphorylation (*26*). Here we demonstrated that this is not the case with respect to NAAPs. Although such a motif (Ser^{246}-Pro-Glu-Lys) exists in the sequence of NAAPs, the kinase does not recognize this sequence as the phosphoacceptor site unless some apparently inhibitory sequences are removed from the native proteins. The present study presents the possibility that the tertiary structure is such that it shields the consensus sequence, thereby preventing access of the kinase. The domain which is involved in preventing access of the kinase to the consensus phosphoacceptor site remains to be determined. Another sequence that has been shown to be phosphorylated by p34^{cdc2}/cyclin B is Ser-X-Y-Arg (where X and Y are any amino acids) of myosin II regulatory light chain (*28*), vimentin (*29*), and other intermediate filaments (*30*). Although one such sequence, Ser^{152}-Lys-Lys-Arg, is present in the sequence of NAAPs, p34^{cdc2}/cyclin B did not catalyze the phosphorylation of this site (Figure 10C). Although the sequence Ser/Thr-Pro has been proposed as a consensus sequence for p34^{cdc2}/cyclin B (*31*) this site is not always phosphorylated (*26*, *32*). It was found in the present study that, when native NAAPs were used as the substrate, the Ser residue in the sequence of Ser^{144}-Pro-Asp-Leu, but not of Ser^{87}-Pro-Leu-Asp, was phosphorylated by p34^{cdc2}/cyclin B (Figure 10B,C). These observations demonstrate that a Pro residue plays an important role as a determinant of specificity for p34^{cdc2}/cyclin B when the succeeding two amino acid residues and the tertiary structure of the substrate protein are favorable for phosphorylation.

A conspicuous feature of NAAPs is that they are located in the nucleus, especially in the nucleolus (*18*), of the embryonic cells. In the amino acid sequence of NAAPs, a putative bipartite nuclear localization signal, Lys^{151}-Ser-Lys-Lys-Arg-Pro-Leu-Ser-Thr-Ser-Asp-Gly-Thr-Ala-Lys-Lys-Thr-Lys, can be found. In experiments not presented here, a cDNA construct encoding a fusion protein which contains cytoplasmic pyruvate kinase, the putative nuclear localization signal of NAAPs, and green fluorescent protein was microinjected into the germinal vesicle of starfish oocytes and localization of the expressed protein was monitored by fluorescence microscopy. It was found that the bipartite nuclear localization signal is essential for nuclear translocation. These findings suggest that the bipartite nuclear localization signal in NAAPs plays a role in determining the nucleus-specific topogenesis of NAAPs.

NAAPs showed a relatively high similarity (overall 41%) to p62 of the sea urchin (*Strongylocentrotus purpuratus*) eggs and embryos (*23*). The protein p62 is associated with the mitotic apparatus and is important for the progression of mitosis in sea urchin embryos. In addition, it is a substrate for Ca^{2+}/calmodulin-dependent protein kinase which is associated with p62 in the mitotic apparatus. When the p62 sequence was examined for consensus nuclear localization signals, none were found (*23*), in contrast to the sequences of NAAPs. The protein p62 was shown to interact directly with chromatin prior to nuclear envelope reassembly, which begins in telophase during mitosis (*23*). In the case of NAAPs, the association of chromosomes was not observed during mitosis of cleaving embryos (data not shown). Moreover, the inclusion of calmodulin together with Ca^{2+} in the reaction mixture inhibited phosphorylation of NAAPs. Therefore, it seems unlikely that NAAPs are functional homologues of p62. Although NAAPs showed a relatively low similarity to NO38 and nucleolin (overall 29.5 and 28.4%, respectively), the major DNA- and RNA-binding nucleolar proteins in the oocyte of the amphibian *Xenopus laevis* (*33*−*36*), there are several characteristics which are common with NAAPs. NO38 and nucleolin contain bipartite nuclear localization signals (*19*, *35*). Furthermore, it has been demonstrated that NO38 and nucleolin are phosphorylated during mitosis and are readily phosphorylated by highly purified starfish p34^{cdc2}/cyclin B in vitro (*32*). Moreover, tryptic phosphopeptide mapping revealed that cdc2 kinase phosphorylates many of the same sites in vitro which are specifically phosphorylated during mitosis in vivo. It follows therefore that NO38 and nucleolin are direct in vivo substrates of mitotically activated cdc2 kinase (*32*). NO38

is thought to play a role in the assembly and transport of ribosomal proteins and preribosomal particles (*37*, *38*). It is conceivable, therefore, that phosphorylation may modulate the interactions between NO38 and either ribosomal RNA or proteins involved in preribosomal particle transport and assembly (*32*). It is thought that nucleolin binds to nascent ribosomal RNA (*36*, *39*) and nucleolar chromatin (*40*) and thus may contribute to the regulation of transcription of ribosomal RNA genes by RNA polymerase I (*41*). Mitotic phosphorylation may thus affect its interactions with rRNA transcripts or with rDNA templates (*32*). With respect to echinoderms, a phylum that has provided a paradigm for maternal ribonucleoproteins for several decades (*42*), molecular studies of DNA-, or RNA-associating proteins are limited. In this study, we report the occurrence of two nucleic acid associated proteins in starfish oocytes. This study focuses on the primary amino acid sequence of two related nucleic acid binding proteins and their posttranslational modifications. The nucleolus of the starfish oocyte, contains a relatively high level of NAAPs. Therefore, it is not unlikely that NAAPs play a functional role in the assembly and processing of preribosomal particles as has been suggested for the amphibian proteins. Further studies are obviously required in order to understand the precise functions of NAAPs in the starfish oocyte. On the basis of the data presented in this and the previous papers (*18*), experiments aimed at a more precise analysis of specific binding interactions between NAAPs and other nucleolar components, particularly rDNA, rRNA, and protein constituents of a nucleolar structure, are currently in progress.

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BI992759X