The Mitotic Apparatus-associated 51-kDa Protein from Sea Urchin Eggs Is a GTP-binding Protein and Is Immunologically Related to Yeast Polypeptide Elongation Factor 1α*

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We investigated the biochemical characteristics of the 51-kDa protein that is a major mitotic apparatus-associated basic protein of sea urchin eggs (Toriyama, M., Ohta, K., Endo, S., and Sakai, H. (1988) Cell Motil. Cytoskeleton 9, 117-128). The amino acid composition of the 51-kDa protein was apparently different from those of tubulin, actin, histones, and myelin basic protein; yet it was similar to those of polypeptide elongation factors 1α (EF-1α). In addition, antibody to EF-1α from yeast cross-reacted with the 51-kDa protein. [3H]-GTP binding activity was detected in the phosphocellulose-purified fraction (PC fraction) which predominantly contained the 51-kDa protein and was shown to be specific to GTP, GDP, guanylyl imidodiphosphate, and ITP. Photo-affinity labeling using [32P]azidoguanosine triphosphate (8-azido-GTP) demonstrated that a 51-kDa polypeptide in the PC fraction specifically bound 8-azido-GTP. This GTP-binding polypeptide was bound to a GTP affinity column, could be eluted by the addition of GTP, and was immunoreactive with anti-51-kDa protein antibodies. When the PC fraction was applied to a gel filtration chromatography column, GTP binding activity was completely coeluted with the 51-kDa protein. Furthermore, the PC fraction and the gel filtration-purified fraction had EF-1α activity: [32P]Pho-tRNA transferring activity to ribosomes in the presence of poly(U) and ribosome-dependent GTPase activity. The results indicate that the mitotic apparatus-associated 51-kDa protein is a GTP-binding protein and suggest that it is structurally and functionally related to yeast EF-1α.

The mitotic apparatus is an indispensable cell organelle in the distribution of chromosomes into daughter cells in eukaryotes, and microtubules play essential roles in the formation of the mitotic apparatus. The centrosomes mainly govern the temporal and spatial arrangement of astral and spindle microtubules, and the kinetochores help organization of the spindle by capturing microtubules from the poles. In addition to these structural elements, Mitchison and Kirschner (1-4) pointed out the importance of the guanine nucleotide-dependent behavior of microtubules (dynamic instability) in the formation of the mitotic apparatus. However, the precise molecular mechanisms of the formation of the mitotic apparatus have not been fully resolved yet.

The 51-kDa protein is a major nontubulin protein component in the mitotic apparatus of sea urchin eggs (5-8). The 51-kDa protein was first demonstrated to be closely correlated to the nucleation of astral microtubules induced by microtubule-organizing granules (MTOGs) in vitro (5, 6). The aster-forming activity of MTOGs could be solubilized from the isolated mitotic apparatus fraction in a solution containing 0.6 M KCl and 50% glycerol. When the extract was dialyzed against a low ionic strength solution, granular assemblies which could form asters when incubated with tubulin at 37 °C were formed. Phosphocellulose column chromatography enabled us to separate the aster-forming protein fraction which contained the 51-kDa protein as a major component. Using immunoaffinity isolation, the 51-kDa protein was shown to be localized in the mitotic apparatus, especially in the centrosomes, the spindle, and the basal region of the asters (7). Monoclonal antibody to the 51-kDa protein, which did not inhibit the aster forming activity of MTOGs in vitro, totally blocked the formation of the mitotic apparatus when antibody was injected into the living eggs before prophase. If antibody was injected at prometaphase, an extremely short spindle (birefringent mass) appeared, causing failure of nuclear division as well as cytokinesis (6, 8). These results indicated that the 51-kDa protein is an essential regulator in the formation of the mitotic apparatus of sea urchin eggs. In this study, we investigate the biochemical characteristics of the 51-kDa protein and show that this protein is a GTP-binding protein structurally and functionally related to polypeptide elongation factor 1α.

MATERIALS AND METHODS

Buffers—PEM1 contained 50 mM Pipes (pH 6.8), 0.5 mM EGTA, 0.25 mM MgCl2, and 50% (v/v) glycerol. PEM2 contained 5 mM Pipes (pH 6.8), 0.5 mM EGTA, 0.25 mM MgCl2, and 50% (v/v) glycerol. PEM1 contained 10 mM Pipes (pH 6.8), 1 mM EGTA, and 0.5 mM MgCl2. PEM2 contained 20 mM Pipes (pH 6.8), 1 mM EGTA, and 0.5 mM MgCl2. PEM3 contained 50 mM Pipes (pH 6.8), 0.5 mM EGTA, 10 mM MgCl2, and 0.1 M KCl.

Preparation of 51-kDa Protein—The 51-kDa protein was prepared as described previously (9, 10).

1 The abbreviations used are: MTOG(s), microtubule-organizing granule(s); EF(s)-1α, elongation factor(s) 1α; EGTA, ethylenebis(oxyethylenetetraacetic acid); GMP-PNP, guanylyl imidodiphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid.

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from whole egg homogenates which were obtained from *Hemicentrotus pulcherrimus* and *Pseudocentrotus depressus* metaphase eggs as described (5). As a partial purification step of the 51-kDa protein, phosophocellulose column chromatography was performed as follows. The Triton X 100 insoluble precipitate of the homogenate was extracted with 0.2 M KCl in PEMG1 for 20 min at 0 °C. The extract was centrifuged at 100,000 × g for 1 h at 0 °C. The supernatant was diluted with an equal volume of PEMG1 to reduce the KCl concentra-
tion to 0.1 M and was centrifuged at 100,000 × g overnight. The supernatant was used for purification of the 51-kDa protein by phosphocellulose column chromatography and GTP affinity chroma-
tography (see below) and was applied to a phosphocellulose column previously equilibrated with PEMG1 containing 0.1 M KCl. After extensive washing with PEMG1 containing 0.5 M KCl, proteins including the 51-kDa protein were washed with PEMG1 containing 0.55 M KCl. We refer to the resulting fraction as the phosphocellulose-purified fraction (PC fraction) and used it for the most part except for amino acid analysis.

The purified 51-kDa protein for amino acid analysis was prepared from the fraction partially purified by phosphocellulose column chroma-
tography from *P. depressus* eggs as described (5). Briefly, the phosphocellulose-purified fraction was dialyzed against PEMG2 over-
night at 0 °C, followed by centrifugation at 100,000 × g for 1 h. To the precipitate was added PEMG2 containing 0.6 M KCl to extract the 51-kDa protein, followed by centrifugation at 100,000 × g for 1 h. The supernatant was applied to a hydroxyapatite column pre-equilib-
ibrated with 0.6 M KCl. The eluate containing 0.6 M KCl was collected. The eluate was dialyzed against 0.55 M KCl. The dialyzed fraction contained highly purified 51-kDa protein.

**Amino Acid Analysis**—The hydroxyapatite-purified 51-kDa protein was concentrated by Amicon YM-05 and dialyzed against 0.2 M (NH$_4$)$_2$CO$_3$. The dialyzed sample was lyophilized and hydrolyzed in 6 M HCl and 4% thioglycolic acid in evacuated sealed tubes for 22 h (duplicate) and 72 h at 110 °C. After evaporation, the amino acids were analyzed on a Hitachi 830 automated analyzer.

**Chromylotropic Digestion**—Digestion of proteins by chymotrypsin (type VII; Sigma) was carried out at 25 °C in 100 μl of the following mixture: 50 μl of the PC fraction containing 50% glycerol and 50 μl of 0.5% chymotrypsin in PEMG1. Samples were soluble until 120 min, and 30, 90, and 120 min after mixing were added with a 1/6 volume of 100 mM phenethylmethylsulfonil fluoride to terminate the reaction. Digested proteins were analyzed by SDS-PAGE and immunoblotting.

**GTP Binding Assays**—The formation of the binary complex of protein with [H]$^3$GTP was followed at 18 °C in a reaction volume of 60 μl (0.1% of the protein fraction containing 50% glycerol and 0.2 M KCl) ± 30 μl of PEMG2 containing 0.5-7.6 μM [H]$^3$GTP (16-17 kCi/mol) ICN and 1 mg/ml bovine serum albumin). Sometimes we used a 50-μl system with the same composition as described above. After 12 min, the reaction mixtures were applied to a nitrocellulose mem-
brane (0.22 μm; Bio-Rad) presoaked in PEMG1. Membranes were washed three times with 3 ml of ice-cold PEMG1, directly dissolved in 1 ml of ACS-II scintillation fluid, and counted by a Packard Tricarb 2200CA liquid scintillation counter. Preincubations with 2 mM GDP, GDP, GMP-PNP, ITP, ATP, CTP, and UTP were done at 0 °C for 30 min.

**Photaffinity Labeling**—Formation of the binary complex of proteins with [α-32P]$^3$GTP or [γ-32P]$^3$GTP from pig liver GTPase was performed by the same method as described above. The final concentrations of [α-32P]$^3$GTP or [γ-32P]$^3$GTP were 5 μM and 0.5 mM. After photolysis, the membranes were solubilized in 0.14 M Tris-HCl (pH 8.0), 4.6% SDS, 25% glycerol, 0.003% pyronin Y, and 10% &mercaptoethanol (SDS sample buffer). Proteins were separated by SDS-PAGE and autoradiography of the dried slab gels. Preincubation of proteins with [α-32P]$^3$GTP or [γ-32P]$^3$GTP was performed by the same method of Bradford (15). SDS-PAGE was performed according to the method of Laemmli (16). After electrophoresis, a gel was stained with Coomassie Brilliant Blue R-250. Immunoblotting was performed by the method of Towbin et al. (17). Anti-51-kDa protein antibodies were prepared as described elsewhere (7). Anti-51-kDa protein antibodies were used for the screening of yeast EF-la cDNA by Nagata (18) and worked as well for pig liver EF-la.*

**GTPase Activity**—Reaction mixtures (50 μl) contained 50 mM Tris-
HCl (pH 7.5), 10 mM Mg(CH$_2$COO)$_2$, 20 mM KCl, 10 mM β-mercapto-
ethanol, 10% glycerol, 0.01 mM GTP containing [γ-32P]GTP (final concentra-
tion of 1 μM), 25 pmol of EF-la or 10 pmol of the 51-kDa protein in the PC fraction, and the indicated amounts of yeast ribosomes, polypeptide, and Phe-tRNA prepared as described below (12). After incubation for 30 min at 30 °C, the mixture was processed according to the method of Conway and Lipmann (19) slightly modi-

cified as follows. The reaction was terminated by adding 125 μl of 0.02 M silicic acid in 0.2 M H$_2$SO$_4$ to the mixture. The mixture was added to 25 μl of 5 M sodium phosphate (pH 7.0) as a carrier of liberated GTP, and 50 μl of 5% (v/v) ammonium molybdate in 4 N H$_2$SO$_4$ to make the phosphomolybdate complex. The yellow complex was extracted with 360 μl of water-saturated 2-butanol/benzene (13, 1/v), and Cerenkov radiation of $^3$P in the organic solvent phase was counted.

**Preparation of Anti-yeast EF-la Antibody**—Anti-EF-la antibodies were prepared in a rabbit in the form of polyclonal antibodies. An EF-la antiserum was used for the screening of yeast EF-la cDNA by Nagata et al. (14).

**Miscellaneous**—Protein concentration was determined by the method of Bradford (15). SDS-PAGE was performed according to the method of Laemmli (16). After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250. Immunoblotting was performed by the method of Towbin et al. (17), and detection of positive immunoreaction was by horse-

radish peroxidase-linked second antibodies (Cappel) at 1:1,000 dilution and by 4-chloro-1-naphthol. Anti-51-kDa protein antibodies were prepared as described elsewhere (7, 8). Antiyeast EF-la serum was added to mitochondria by incubation at 37 °C with 0.5 mg of EF-la from Saccharomyces carlsbergensis three times with Freund's complete adjuvant. First and second injections were made subcutaneously at 2-week interval. Seventeen days after the second injection, the last injection was made intramuscularly, and the rabbit was bled 2 weeks after the last injection. The antiserum was subjected to ammonium sulfate precipitation (30 and 40% successively) and stored at −20 °C. This antiserum was used for the screening of yeast EF-la cDNA by Nagata et al. (14).
absorption experiment, the FC fraction was electrophoresed, and the 51-kDa protein band was cut off and homogenized in 20 mM phosphate/KOH (pH 7.4), 150 mM NaCl containing 3% (w/v) bovine serum albumin (Sigma). Anti-EF-1α antiserum was diluted (1:100) in this gel homogenate and incubated overnight at 0 °C. The mixture was centrifuged at 30,000 × g for 20 min, and the supernatant was used as the absorbed antiserum. Aster forming activity was measured as described elsewhere (18), and isolation of the mitotic apparatus from H. pulcherrimus was performed as described (6, 18).

RESULTS

Amino Acid Composition of 51-kDa Protein—To investigate chemical properties of the 51-kDa protein, we purified the 51-kDa protein as described under “Materials and Methods.” Most of the 51-kDa protein did not bind to the hydroxylapatite column (Fig. 1D, lanes FT), in contrast with other proteins which could be eluted by the addition of 0.6 M KH₂PO₄. Since the 51-kDa protein in the flow-through fractions was close to homogeneity, we used these fractions for amino acid analysis. We could obtain 0.88 mg of the 51-kDa protein from 50 ml of packed sea urchin egg cells.

The amino acid composition of the 51-kDa protein from P. depressus is shown in Fig. 1 together with those of chicken β-tubulin (19), rabbit skeletal actin (29), myelin basic protein (21), histone H1 (22), and human (23) and yeast (14, 24, 25) EFs-1α. The amino acid composition of the 51-kDa protein was plotted in the order of the molar content of each amino acid residue. Corresponding molar contents of the other proteins were also plotted on Fig. 1. EF-1α is the α-subunit of elongation factor 1 which is involved in the binding of aminoaeryl-tRNAs to 80 S ribosomes during the elongation of the polypeptide chain (26–28). Unfortunately, the amino acid composition of EF-1α from sea urchin is not known because EF-1α from sea urchin eggs was not identified yet. However, the amino acid sequences of EFs-1α which were shown to be highly conserved (80–90%) (14, 23–25, 29) enabled us to compare the 51-kDa protein with EFs-1α. The 51-kDa protein

![Fig. 1. Comparison of amino acid composition of 51-kDa protein with those of several proteins. The 51-kDa protein from P. depressus was purified by hydroxylapatite column chromatography, and the amino acid composition of the purified 51-kDa protein (●) was analyzed as described under "Materials and Methods." Relative amounts of amino acids (mole percent) were plotted in the order of the molar content of each amino acid residue. A, comparison of amino acid compositions of the 51-kDa protein, β-tubulin, and actin; B, comparison of the 51-kDa protein, myelin basic protein, and histone H1; C, comparison of the 51-kDa protein and EFs-1α from human and yeast. Amino acid compositions of β-tubulin (●) and of EFs-1α from human (○) and yeast (○) are from the sequences deduced from cloned cDNA (tubulin (19), human EF-1α (29), and yeast EF-1α (14, 24, 25)). Data of actin (○), myelin basic protein (□), and histone H1 (■) are from Refs. 20–22, respectively. D, purification of the 51-kDa protein by hydroxylapatite column chromatography. Lanes FT indicate the purified 51-kDa protein in the flow-through fractions of hydroxylapatite column. Adsorbed proteins were eluted by the addition of 0.6 M KH₂PO₄. The arrowhead indicates the 51-kDa protein. Molecular weights are indicated in thousands.](http://www.jbc.org/bc)
had a higher content of lysine (10.9 mol %) and valine (9.4 mol %). This feature was distinct from other cytoskeletal proteins (tubulin and actin) and some basic proteins (histones and myelin basic protein), but was similar to EFs-1α. The greater number of lysine residues implies the basic property of both proteins. Actually, the isoelectric points of the 51-kDa protein (9.8) and EFs-1α (8.5–9.5) are considerably high. Furthermore, EFs-1α have molecular weights (49,000–53,000) similar to that of the 51-kDa protein. Despite the similarity of the contents of most of the amino acid residues between the 51-kDa protein and EFs-la, some differences were observed, i.e. the 51-kDa protein has a greater number of serine residues and a lower number of threonine and aspartic acid + asparagine residues than do EFs-1α.

Antiserum to yeast (S. carlsbergensis) EF-la cross-reacted with the isolated mitotic apparatus fraction (lane 4') from H. pulcherrimus eggs. The antigenic fraction (lane 1'), the isolated mitotic apparatus fraction (lane 2'), the PC fraction (lane 3'), and the gel filtration-purified fraction (lane 4') from H. pulcherrimus eggs. The antigenic polypeptide coincided with the 51-kDa protein detected by monoclonal anti-51-kDa protein antibody HP1 (lanes 1'–4'). When anti-EF-la antiserum was absorbed by the 51-kDa protein, immunoreaction was greatly reduced.

Anti-EF-la antibody affinity-purified by the 51-kDa protein from H. pulcherrimus (aEFap51) cross-reacted only with the protein band corresponding to the 51-kDa protein in the whole egg homogenate (Fig. 2C, lane 5'). In addition, aEFap51 and anti-51-kDa antibody HP1 were also shown to react with EF-1α from S. carlsbergensis. Immunofluorescence labeling of the sections of paraffin-embedded H. pulcherrimus eggs by aEFap51 revealed the same localization pattern of the 51-kDa protein in the mitotic apparatus (Fig. 2B) as that detected by monoclonal and polyclonal antibodies against the 51-kDa protein (7). Anti-EF-la antiserum presented a similar staining. When we used antiserum absorbed by the 51-kDa protein, labeling of the mitotic apparatus was largely reduced. No significant labeling was detected in the control experiments using nonimmunized γ-globulin or second antibody only. These results indicated that the 51-kDa protein has at least one common antigenic determinant with EF-1α of S. carlsbergensis EF-la. Furthermore, limited chymotryptic digestion of the 51-kDa protein left a stable 43-kDa polypeptide whose molecular mass was similar to those of EFs-la previously reported (12, 30–33). From these, we concluded that the 51-kDa protein shares some structural resemblance with EF-1α.

51-kDa Protein Binds GTP—During binding of aminoacyl-tRNAs to 80 S ribosomes, GTP is hydrolyzed into GDP by EF-1α (28). To perform this function, EF-1α has the guanine nucleotide-binding site and the GTPase domain whose amino acid sequences are homologous to those of elongation factors Tu and G, initiation factor 2, G-proteins, and RAS proteins (23). In addition, EF-1α interacts with EF-1β to exchange bound GDP for GTP (34). Thus, EF-1α shares a common property with G-proteins.

From the structural resemblance described above, it was plausible that the 51-kDa protein binds GTP as does EF-1α. Therefore, we tested the possibility of GTP binding to the 51-kDa protein. We had succeeded in purifying the 51-kDa protein by hydroxylapatite column chromatography as described before (Fig. 1D), so we examined whether or not the 51-kDa protein in this fraction could bind [³H]GTP. Unfortunately, the hydroxylapatite-purified 51-kDa protein did not bind [³H]GTP. When we digested the hydroxylapatite-purified 51-kDa protein with chymotrypsin, it could no longer generate the stable 43-kDa polypeptide described before, although the 51-kDa protein in this fraction was immunoreactive with all anti-51-kDa protein antibodies. Therefore, we considered that the hydroxylapatite-purified 51-kDa protein was denatured by an irreversible conformational change. Then, we studied the binding of GTP to the 51-kDa protein fractionated by phosphocellulose column chromatography. We found that proteins in 30 μl of the PC fraction (containing 17 pmol of the 51-kDa protein) bound 13 pmol of [³H]GTP, showing a ratio of 1:0.76. This suggested that 1 mol of the 51-kDa protein would intrinsically bind 1 mol of GTP. When the 51-kDa protein bound to the phosphocellulose column was eluted by a linear concentration gradient of KCl, the elution of the GTP binding activity was superimposed with that of the 51-kDa protein.

[³H]GTP binding was greatly reduced by preincubation of the PC fraction with 2 mM cold GTP, GDP, GMP-PNP, and ITP, but not with 2 mM ATP, UTP, and CTP (Fig. 3). GMP-PNP and ITP are structural analogues of GTP; therefore, binding is reasonable. This binding specificity was similar to
binding of \([3H]\)GTP was measured by the nitrocellulose filtration method described under "Materials and Methods." \([3H]\)GTP bound with \([3H]\)GTP after preincubations with 2 mM cold GTP, GDP, ATP, UTP, and CTP at 0 °C for 30 min; and binding of \([3H]\)GTP was measured by the nitrocellulose filtration method described under "Materials and Methods." \([3H]\)GTP bound to the fraction without preincubation (13 pmol) was expressed as 100%.

 Autoradiography (lanes 1'-5') revealed the existence of photolabeled polypeptides. We detected 51-, 42-, and 41-kDa polypeptides which incorporated \([\gamma-32P]\)8-azido-GTP (lane 1'). Incubation of the PC fraction with \([\gamma-32P]\)8-azido-GTP (lanes 2 and 2') and 2 mM ATP (lanes 3 and 3') was done as described under "Materials and Methods." Lanes 4 and 4' indicate a control without photolysis. Arrows show the position of the 51-kDa protein. Molecular weights are indicated in thousands.

The most prominent polypeptide had a M, of 51,000. A 45-kDa polypeptide was also present. Immunoblotting of this fraction using the anti-51-kDa protein monoclonal (HP1) and polyclonal (AP17) antibodies (Fig. 5A, lanes HP1 and AP17) demonstrated that the 51-kDa polypeptide was the 51-kDa protein. We examined the aster forming activity of this GTP eluate according to the method reported previously (5, 18). Unfortunately, the aster forming activity was lost after dilution of glycerol. When the concentration of glycerol is decreased below 50%, the aster forming activity becomes quite labile (5, 18). The 51-kDa protein in the fraction from the purification step just before phosphocellulose chromatography could not fully combine with GTP-Sepharose (lane ET). The yield of the 51-kDa protein was 40% of the initial amount of the 51-kDa protein from densitometric analyses. This may be due to denaturation by low concentrations of glycerol. The 45-kDa polypeptide in the same fraction was shown not to bind \([P\]8-azido-GTP (data not shown), and so the 51-kDa protein did not bind to GTP-Sepharose via the 45-kDa polypeptide. The 45-kDa polypeptide was not a kind of degradation product of the 51-kDa protein from immunoblotting. The 45-kDa polypeptide may be a protein which binds to the 51-kDa protein.

We further tested whether or not the 51-kDa protein in the PC fraction could bind to GTP-Sepharose (Fig. 5B). The phosphocellulose-purified 51-kDa protein seemed to bind to GTP-Sepharose more tightly because a higher concentration of GTP (6 mM) or KC1 (1 mM) was necessary for elution of the bound 51-kDa protein. The yield of the 51-kDa protein was 30%. In this case, no apparent polypeptide could be seen other than the 51-kDa protein eluted by GTP or KC1 (lanes 7 and 11), ruling out the possibility that the 51-kDa protein bound via another protein that binds GTP.

In addition to these experiments, we fractionated the PC fraction by Sephacryl S-300 gel filtration column chromatography and studied whether or not GTP binding activity coeluted with the 51-kDa protein (Fig. 6). Elution of the 51-kDa protein and GTP binding activity coincided perfectly. The peak fractions, 42-44, predominantly contained the 51-kDa protein (70% at the maximum level from densitometric analysis). The ratio of the 51-kDa protein to bound GTP was ~1:0.7-0.8, again suggesting that the 51-kDa protein intrin-
chromatography. A, affinity purification of the 51-kDa protein in between bovine serum albumin and ovalbumin. From the data showing good agreement with the yield of GTP binding activity (Fig. 7A, bar b) the position of the 51-kDa protein. Molecular weights are indicated second by PEM3 containing 5% glycerol and 1 M KCl. Lane PC, the addition of PEM3 containing 5% glycerol and 6 mM GTP and PEM3 containing 5% glycerol and 0.3 M KCl. Proteins in each fraction were analyzed by SDS-PAGE (lanes S, FT, and 8–16) and by immunoblotting (lanes HP1 and AP17). Lane S, applied sample; lane FT, flow-through fraction; lanes 8–16, eluates; lanes HP1 and AP17, immunoblotting of fraction 9 by monoclonal (HP1) and polyclonal (AP17) anti-51-kDa protein antibodies, respectively. The arrow indicates the position of the 51-kDa protein. B, affinity purification of the 51-kDa protein in the PC fraction. The PC fraction (1.1 mg/ml, 0.5 ml) was diluted 10 times with PEM3 and applied to GTP-Sepharose as described above. After washing, the bound 51-kDa protein was eluted first by the addition of PEM3 containing 5% glycerol and 2 mM GTP and PEM3 containing 5% glycerol and 0.3 M KCl. Proteins in each fraction were analyzed by SDS-PAGE (lanes S, FT, and 8–16) and by immunoblotting (lanes HP1 and AP17). Lane S, applied sample; lane FT, flow-through fraction; lanes 8–16, eluates; lanes HP1 and AP17, immunoblotting of fraction 9 by monoclonal (HP1) and polyclonal (AP17) anti-51-kDa protein antibodies, respectively. The arrow indicates the position of the 51-kDa protein. Molecular weights are indicated in thousands.

FIG. 5. Affinity purification by GTP-Sepharose column chromatography. A, affinity purification of the 51-kDa protein in the fraction just before the phosphocellulose chromatography step. Preparations of GTP-Sepharose and the fraction applied were performed as described under “Materials and Methods.” The column (0.8 ml) was pre-equilibrated with 20 ml of PEM3 containing 5% glycerol at 4 °C, and 30 ml of the fraction diluted 10 times (containing 1.5 mg of proteins) was applied. After washing with 3 ml of PEM3 containing 5% glycerol, elution was performed successively by PEM3 containing 5% glycerol and 2 mM GTP and PEM3 containing 5% glycerol and 0.3 M KCl. Proteins in each fraction were analyzed by SDS-PAGE (lanes S, FT, and 8–16) and by immunoblotting (lanes HP1 and AP17). Lane S, applied sample; lane FT, flow-through fraction; lanes 8–16, eluates; lanes HP1 and AP17, immunoblotting of fraction 9 by monoclonal (HP1) and polyclonal (AP17) anti-51-kDa protein antibodies, respectively. The arrow indicates the position of the 51-kDa protein. Molecular weights are indicated in thousands.

FIG. 6. Sephacryl S-300 gel filtration chromatography of PC fraction. Gel filtration was performed on a column (1.3 X 21.5 cm) of Sephacryl S-300 in PEMG1 containing 0.56 M KCl. The PC fraction (0.56 mg/ml, 1.5 ml) was applied to the column, and 0.65 ml of each fraction was collected. [3H]GTP binding was measured using 30 ml of the column fraction as described under “Materials and Methods.” Protein concentration was determined by the method of Bradford (15). The amount of the 51-kDa protein in the assay mixture was calculated from the densitometric data. The protein compositions of the peak fractions for [3H]GTP binding are shown in the inset with fraction numbers. The arrowhead indicates the 51-kDa protein.

1 as a reference for assays. Marked [4C]Phe-tRNA binding activities were found in fractions containing the 51-kDa protein: the PC fraction (Fig. 7A, bar b) and the gel filtration-purified fraction (bar f). We adjusted the amount of the 51-kDa protein and yeast EF-1α to ~3 pmol in the reaction mixtures. The activities of these fractions were 60% (the PC fraction) and 53% (the gel filtration-purified fraction) of the activity of yeast EF-1α, as normalized to the amount of the 51-kDa protein in both fractions. Other fractions from gel filtration (bars c–g) presented no activity.

In addition, the PC fraction revealed ribosome-dependent GTPase activity (Fig. 7B, bar h), as did yeast EF-1α (bar d). We estimated that the PC fraction had 50% (0.26 mol/mol/min) of the yeast EF-1α GTPase activity in the presence of ribosomes, Phe-tRNA, and poly(U). If ribosomes and/or Phe-tRNA was absent, GTPase activities of both yeast EF-1α (bars a–c) and the PC fraction (bars e–g) were reduced. In contrast with yeast EF-1α, it seemed that the PC fraction had slight GTPase activity independent of ribosomes and Phe-tRNA (bars e–g). These results suggest that the 51-kDa protein is functionally related to EF-1α.

DISCUSSION

The results of this paper show that the mitotic apparatus-associated 51-kDa protein is a GTP-binding protein which is structurally and functionally related to EF-1α. The PC fraction had [3H]GTP binding activity suited for the amount of
ity chromatography, and gel filtration show that the 51-kDa protein specifically binds GTP with a stoichiometry of 1:1. Preliminary results indicated that the relative amount of the 51-kDa protein in the soluble fraction was less than 0.1% of the total protein, in contrast to the high content of EF-1α in the soluble fractions of other eukaryotic cells (~5% (33, 36)).

Even though sea urchin eggs were treated with 100 μg/ml puromycin just after the streak stage, mitotic apparatuses which were functionally and morphologically normal were formed, and the eggs were normally divided (data not shown). In contrast, microinjection of monoclonal anti-51-kDa antibody into the eggs at the same stage mostly blocked mitosis, which most likely was caused by the formation of an abnormal spindle (8). Since EF-1α from sea urchin eggs have not been identified and purified, we cannot precisely compare the 51-kDa protein with authentic egg EF-1α. Further investigations, such as determination of primary structure, purification of sea urchin egg EF-1α, followed by comparison with the 51-kDa protein, and study of the effect of post-translational modification, should be performed for potent explanations of the relationship between them.

EF-1α has many homologues. There have been several reports on protein species homologous to EF-1α. Kikuchi et al. (37) reported a gst1 mutant of Saccharomyces cerevisiae. At nonpermissive temperature, mutant cells with large buds accumulated, and DNA synthesis was substantially arrested, in contrast to no suppression of protein synthesis. The mutation was suggested to affect the G1-to-S phase transition in the cell cycle. The gene product (M, 76,565) has a portion that is highly homologous (codon 254 to the carboxyl terminus) to yeast EF-1α (40% in the nucleotide sequence). Mattaj et al. (38) reported that 425p48, the most abundant soluble protein of previtellogenic Xenopus oocytes, is immunologically and functionally related to EF-1α. This protein is a component of the 42 S ribonucleoprotein particle and binds to tRNA. They discussed that 425p48 is a stage-specific elongation factor. Kuriyama et al. (39) showed that monoclonal antibody (SU5), prepared from isolated mitotic apparatuses of sea urchin eggs preferentially stained the centrosomes and recognized a 30-kDa polypeptide on immunoblotting. Furthermore, the same group recently reported that a 34-kDa portion of the centrosphere-associated 50-kDa antigen has an amino acid sequence highly homologous (70-80%) to those of EF-1α from yeast, shrimp, and human and the “female factor” of Drosophila melanogaster (40). Furthermore, we have most recently found that the isolated centrosomes and the mitotic spindles from Chinese hamster ovary cells contain 49-kDa basic protein which is immunoreactive with both the anti-centrosome antibody from a scleroderma patient and αEFap51. The relationship of the 51-kDa protein to these proteins remain to be solved after determination of the amino acid sequence of the 51-kDa protein.

The 51-kDa protein has been demonstrated to have important contributions to the construction of the mitotic apparatus. It was suggested that the 51-kDa protein is involved in the aster forming activity of MIUGs in vitro (5) and in mitotic apparatus formation in vivo (7, 8). This is the first report that demonstrates the intrinsic property of GTP binding of a protein other than tubulin in the mitotic apparatus. GTP-binding proteins, such as G-proteins, RAS protein, and elongation factors have the common property that the GTP form is active and the GDP form is inactive (28, 41). Guanine nucleotides play an important role in the formation of microtubules. GTP supports the elongation of microtubules, whereas GDP does not under physiological medium condi-

**Fig. 7.** EF-1α-like activity in 51-kDa protein fractions. A, [14C]Phe-tRNA transferring activity to ribosomes of yeast EF-1α (bar a), the PC fraction (bar b), and gel filtration purified fractions in Fig. 6 (bar c, fraction 28; bar d, fraction 33; bar e, fraction 36; bar f, mixture of fractions 40-44; bar g, fraction 50) was measured as described under "Materials and Methods." The assay was performed at 30 °C with 30 min as described under "Materials and Methods." To EF-1α or the 51-kDa protein, we added 5 μg of poly(U) (bars a and e), 5 μg of poly(U) + 0.5 A260 unit of ribosomes (bars b and f), 5 μg of poly(U) + 50 pmol of Phe-tRNA (bars c and g), or 5 μg of poly(U) + 0.5 A260 unit of ribosomes + 50 pmol of Phe-tRNA (bars d and h). The amino acid composition, the chymotrytic digestion pattern of the 51-kDa protein, and antigenicity to anti-EF-1α antibody showed structural resemblance of the 51-kDa protein to EF-1α. Interestingly, the fractions containing the 51-kDa protein as a main component could transfer charged aminoacyl-tRNA to ribosomes and hydrolyze GTP in a ribosome-dependent manner. Since these fractions did not have any potent GTP-binding proteins other than the 51-kDa protein, it is most likely that the 51-kDa protein can act as EF-1α. Together with the GTP binding ability of the 51-kDa protein, it was suggested that the 51-kDa protein is also functionally related to EF-1α. It seems possible that the 51-kDa protein is EF-1α from sea urchin eggs. However, some discrepancy arises if the 51-kDa protein is EF-1α itself. For instance, the 51-kDa protein was purified from an insoluble fraction of sea urchin eggs and was shown to exist mainly in insoluble components as detected by subcellular fractionation. Preliminary results indicated that the relative amount of the 51-kDa protein in the soluble fraction was less than 0.1% of the total protein, in contrast to the high content of EF-1α in the soluble fractions of other eukaryotic cells (~5% (33, 36)).

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3. K. Ohta, unpublished data.

4. T. Uetsuki, personal communication.

5. K. Ohno, M. Miyazaki, Y. Moroi, S. Endo, and H. Sakai, manuscript in preparation.
tions. In dynamic instability theory, microtubules undergo phase transitions between the growing phase in which GTP subunits associate to microtubule ends and the shrinking phase in which unstable polymers consisting of GDP-tubulin depolymerize at a fast rate following the loss of stabilizing terminal GTP subunits (2-4). Involvement of GTP-binding proteins in the nucleation process of microtubule formation on microtubule organizing centers makes it possible to speculate that the GTP form supports nucleation, but the GDP form does not. The 51-kDa protein was shown to be a component of MTOGs and to be involved in the nucleation of microtubule formation on MTOGs. It is possible that the 51-kDa protein interacts more closely with the tubulin dimer than does the GDP form, proposing a hypothesis that the 51-kDa protein, as a G-protein, initiates assembly of microtubules in vitro. The effect of GTP and GDP on the aster forming activity of MTOGs remains to be solved.

Besides the functional participation of the 51-kDa protein in microtubule nucleation, the 51-kDa protein may have an important role in the formation of spindles in sea urchin eggs. The 51-kDa protein has been shown to localize in the spindle. Microinjection of monoclonal anti-51-kDa antibody into living eggs at prometaphase brought about pronounced shortening of the spindle (8), suggesting that the 51-kDa protein functions as a kind of stabilizer of microtubules in the spindle. Since the 51-kDa protein has been shown not to bind to tubulin on the entire surface of microtubules in vitro from a cosedimentation experiment (7), it is unlikely that the 51-kDa protein acts as a microtubule-associated protein which stabilizes microtubules by direct association. Possibly, the 51-kDa protein may modulate the formation of the mitotic apparatus by a similar mechanism to other GTP-binding proteins in signal transduction. Further investigations need to be made to understand the significance of the GTP-binding 51-kDa protein in the centrosome and the spindle.

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