Molecular Characterization of p62, a Mitotic Apparatus Protein Required for Mitotic Progression*

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A 62-kDa (p62) mitotic apparatus-associated protein is important for the proper progression of mitosis in sea urchin embryos (Dinsmore, J. H., and Sloboda, R. D. (1989) Cell 53, 769–780). We have isolated and characterized a full-length p62 cDNA of 3374 base pairs which encodes an extremely acidic polypeptide of 411 amino acids having a calculated M₆ of 46,388 and a pI of 4.01; p62 is a unique protein with no significant identity to any known proteins. Southern and Northern blot analyses demonstrate that the gene for p62 is present once in the sea urchin genome and the corresponding mRNA is present in unfertilized eggs and in early embryos through and up to the gastrula stage. Sequence analysis suggests certain regions may participate in chromatin association and microtubule binding, an observation that is consistent with previous immunological data (Ye, X., and Sloboda, R. D. (1995) Cell Motil. Cytoskeleton 30, 310–323) as well as data reported herein. Confocal microscopy reveals that during interphase the protein binds to chromatin in the nuclei of sea urchin eggs. In the germinal vesicles of clam oocytes at prophase of meiosis I, p62 binds to the condensed chromosomes. Currently, truncated clones of p62 are being used to identify the tubulin and chromatin binding domains.

Controlled microtubule disassembly occurs during anaphase A, the stage of mitosis characterized by the movement of the chromosomes toward the poles, an event thought to be powered by molecular motors that reside at the kinetochores. The kinetochore microtubules selectively disassemble during anaphase A, and if the microtubules are prevented from disassembling, e.g. by treatment with the microtubule stabilizing, anti-tumor drug Taxol, then mitosis ceases. Microtubule depolymerization can be induced by calcium, and calcium has been shown to result in the shortening of the distance separating the poles and movement of the chromosomes toward the poles (1). Furthermore, microtubule disassembly is necessary for the reactivation of chromosome movement in lysed cells (2), and isolated mitotic chromosomes can be translocated in association with a single microtubule in vitro as the result of microtubule depolymerization (3, 4). Thus, a critical step in anaphase is the controlled disassembly of the kinetochore microtubules.

We have previously demonstrated that a protein of 62 kDa (p62), a substrate of a calcium/calmodulin-dependent protein kinase, is associated with the mitotic apparatus (5, 6). Both p62 and the kinase responsible for its phosphorylation copurify with mitotic apparatuses isolated from first cell cycle sea urchin embryos (5, 7). Importantly, the phosphorylation of p62 has been shown to correlate directly with the disassembly of microtubules in isolated mitotic apparatuses (5). Furthermore, microinjection of sea urchin embryos with affinity-purified antibodies to p62 inhibits progression through the cell cycle, specifically at the metaphase to anaphase transition (6). Because p62 is present at constant levels through at least two cell cycles (8), p62 dephosphorylation must be due to the action of a specific phosphatase and not to the degradation of the protein, as occurs, for example, with the cyclins. In fact, the existence of protein phosphatase 1 in the mitotic apparatus isolated from sea urchin embryos that dephosphorylates p62 has been demonstrated by pharmacological studies with phosphatase inhibitors (9). Finally, the subcellular localization of p62 varies with the cell cycle. During mitosis, p62 is associated with the mitotic apparatus, particularly with the microtubules (6, 8, 10), while during interphase, p62 resides in the nucleus (8, 10).

Based on this background, our working hypothesis is that phosphorylation and dephosphorylation of p62 play key roles in controlling cell division, particularly during anaphase A, by permitting kinetochore microtubule disassembly at the appropriate time during mitosis. As the logical next step in elucidating the mode of action of p62 in mitosis, we present here a characterization of the polypeptide based on its deduced amino acid sequence obtained from cloned cDNA. The p62 polypeptide is highly acidic, yet contains distinct subdomains of alternating clusters of basic and acidic residues. Furthermore, confocal microscopy reveals that during interphase the protein binds to chromatin in the nuclei of sea urchin eggs; in the germinal vesicles of clam oocytes at prophase of meiosis I, p62 binds to the condensed chromosomes.

MATERIALS AND METHODS

Generation of Peptide Fragments of p62 and Protein Microsequencing

Electrospray was used to purify p62, as described previously (10). The purified, denatured protein was concentrated using a single Centricon-10 ultrafiltration device according to the instructions of the manufacturer (Amicon, Inc., Beverly, MA).

CNBr Cleavage—Purified p62 (100 μl) was dialyzed against 0.5 mM NH₄HCO₃ at 4 °C overnight, lyophilized, and resuspended in 150 μl of 50 mg/ml CNBr (Eastman Kodak Co.) in 70% (v/v) formic acid and incubated overnight in the dark at room temperature. An equal volume of deionized water was then added, and the sample was shell-frozen in liquid N₂ and lyophilized. Thirty microliters of reducing buffer (31.25 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.004% pyronin Y) were added, and the sample was boiled for 4 min and resolved by electrophoresis on a 5–15% gradient polyacrylamide SDS-gel (11). The fragments were blotted to polyvinyldene difluoride mem-
brane in CAPS\textsuperscript{1} transfer buffer (10 mM CAPS, 10% MeOH, pH 11.0) at 300 mA at 4 °C for 75 min. The membrane was rinsed with deionized water, and stained for 1 min with Coomassie Blue R-250 in 40% MeOH, 1% acetic acid and destained in 50% MeOH for 1 min followed by rinses with deionized water. Bands of interest were excised and loaded directly onto a sequencing cartridge of an Applied Biosystems-476A protein sequenator.

Tryptsin Digestion—Tryptsin digestion of p62 blotted to Trans-Blot membrane (Bio-Rad) was used in a parallel approach to obtain sequence data from internal regions of p62. After electrophoresis and electroblotting, the membrane was rinsed with Milli-Q water three times for 5 min each, then immersed for 1 min in a buffer containing 0.1% Amido Black in 10% acetic acid and destained for 1 min in 5% acetic acid. The band corresponding to p62 was excised, rinsed with Milli-Q water, air-dried, and sent to the Wistar Protein Microchemistry Laboratory at the Wistar Institute (Philadelphia, PA) where \textit{in situ} proteolytic digestion of p62 with trypsin was performed. Three peptides identified by HPLC from the trypsinic digest were chosen, isolated, and sequenced in a gas phase sequenator.

Sea Urchin cDNA Expression Library and Screening

A sea urchin \textit{ZAP} cDNA library provided courtesy of Dr. Jonathan Scholey (University of California, Davis) was screened (12) with affinity-purified p62 polyclonal antibodies (see Ye and Sloboda (10) for antibody characterization) that had been preabsorbed with an \textit{Escherichia coli} phosphatase lysate. One positive clone, \textit{ZAP} h1, was identified using this procedure. The plIIscript vector, containing the 5′ oligonucleotide removed from the \textit{ZAP} vector by \textit{in vivo} excision, and the resulting clone was named SKh1. As an initial step in clone verification, the cDNA clone encoding p62; RT, reverse transcriptase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; 3A\textsubscript{upper}, sense primer for RT-PCR; 3A\textsubscript{lower}, antisense primer for RT-PCR; 3A, redundant oligomer predicted to be specific for RT-PCR primer pair 3A\textsubscript{upper} and 3A\textsubscript{lower}; HPLC, high performance liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid; CTAB, hexadecyltrimethylammonium bromide; EMAp, echinoderm MAP.

Characterization of a Mitotic Apparatus Phosphoprotein

DNA Sequencing and Analysis; \textit{In Vitro} Transcription and Translation

Plasmid DNA was isolated from recombinant clones using the plasmid mini kit from Qiagen and sequenced via the dideoxy chain termination method on an Applied Biosystems model 373A automated se-

The abbreviations used are: CAPS, 3-(cyclohexylamino)propanesulfonic acid; Cam kinase II, calcium- and calmodulin-dependent protein kinase II; MAP, microtubule-associated protein; SKh11, full-length cDNA clone encoding p62; RT, reverse transcriptase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; 3A\textsubscript{upper}, sense primer for RT-PCR; 3A\textsubscript{lower}, antisense primer for RT-PCR; 3A, redundant oligomer predicted to be specific for RT-PCR primer pair 3A\textsubscript{upper} and 3A\textsubscript{lower}; HPLC, high performance liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid; CTAB, hexadecyltrimethylammonium bromide; EMAp, echinoderm MAP.

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Nucleus Immunolabeling Experiments

Sea urchin nuclei were isolated with membranes intact from *Lytechinus pictus* eggs (8). Germinal vesicles were isolated from oocytes of *the surfclam* *Spisula solidissima* as follows. Oocytes were washed three to four times in seawater. One milliliter of packed oocytes was collected by centrifugation in a clinical centrifuge at maximum speed, washed for 30 s in 1M glycerol, then washed for 60–90 s in 1M glycerol, 10 mM NaH2PO4, pH 8.0. Oocytes were resuspended in 20 volumes of Nonidet P-40 buffer (0.25M sucrose, 50 mM PIPES, pH 6.9, 0.75 mM MgSO4, 10 μg/ml leupeptin, 1 mM dithiothreitol, 160 μM Pefabloc, and 0.5% Nonidet P-40), applied over a 0.4M sucrose cushion, and centrifuged for 10 min in a clinical centrifuge at three-fourths speed. The germinal vesicles in the pellet were washed several times by resuspension and sedimentation through sucrose.

Isolated nuclei or germinal vesicles were fixed in 90% methanol, 50 mM EGTA at 2°C, and processed for indirect double label immunofluorescence (10) using a mixture of affinity purified antibodies to p62 and DNA antibodies, the latter kindly provided by Dr. Robert Goldman (Northwest University Medical School). Secondary antibodies were a mixture of fluorescein-labeled goat anti-human antibodies and rhodamine-labeled goat anti-rabbit antibodies. Slides were viewed using an LSM-410 inverted confocal laser scanning microscope from Carl Zeiss, Inc.

RESULTS

Protein Microsequencing—Purified p62 was cleaved with CNBr to yield two cleavage products, pL and pS, with estimated molecular masses of 36 and 20 kDa, respectively. These peptides were specific to CNBr cleavage as they were absent from control samples which revealed slight degradation of p62 due to formic acid alone. Digestion with CNBr also generated several other peptides that were detected on Coomassie Blue-stained SDS-gels, but these were not efficiently transferred to the polyvinylidene difluoride membrane. When a duplicate gel was transferred to nitrocellulose and probed with affinity purified p62 antibodies, both pL and pS as well as several smaller peptides were recognized by the antibodies. When phosphorylated p62 was cleaved with CNBr, peptides pL and pS were radioactive, indicating they contained one or more residues that could be phosphorylated under the conditions employed in the in vitro phosphorylation reaction of p62 (5). This observation also indicates that p62 is phosphorylated in vitro at more than one site, assuming that the CNBr cleavage reaction went to completion. Peptides pL and pS were sequenced, and five sequential residues from each peptide were obtained with confidence. These sequences were named CNBr-1 (NH2-AKEYF-COOH) and CNBr-2 (NH2-KGKGD-COOH).

Tryptic peptides were generated and purified by HPLC. Three peptides were selected, sequenced, and the resulting peptide sequences were called Trp-1 (NH2-LGLNESTNLDLGQPPVT-COOH), Trp-2 (NH2-NVIEVETINFDGETVIIQPLLSR-COOH), and Trp-3 (NH2-DDDRNVIEVETINFDGETVIIQPLLSR-COOH). Note that Trp-2 is a fragment of Trp-3 formed by cleavage after an Arg residue found at position number four of Trp-3.

Library Screening—Two approaches, an antibody screen and a PCR-based screen, were used simultaneously to identify p62 clones from a sea urchin cDNA library. Both approaches identified the same clone, SKh1, which encodes tryptic peptides Trp-1 and Trp-3, as well as the CNBr-derived peptides, CNBr-1 and CNBr-2, and contains an open reading frame of 225 residues (25 kDa). However, the 225-residue open reading frame extends to the 3' end of the clone, there is no in frame stop codon, poly(A) addition signal, or poly(A) tail. Thus, SKh1 is a partial cDNA clone of p62, and thus could be used to rescreen the cDNA library to identify a full-length clone.

**FIG. 1.** Nucleotide and deduced amino acid sequence of p62 cDNA clone SKh1. The cDNA clone SKh1 was identified from a cDNA library using antibodies and RT-PCR. Shown here is the nucleotide sequence of SKh1 and the corresponding primary sequence. Tryptic peptides Trp-1 and Trp-3 are shown by underlines beneath the deduced amino acid sequence, and the two CNBr peptides are shown in bold type and by underlines beneath the deduced amino acid sequence. Asterisks denote the first in frame stop codon. Three polyadenylation sequences (ATTAAA) are underlined; the putative poly(A) addition signal is the one at nucleotides 3336–3341. GenBank™ accession no. U76750.
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Adenylation signals are most likely not used, at least by the transcript that generated clone SKh11, as they are >35 bases upstream of the poly(A) tail. Furthermore, no correspondingly smaller species of mRNA were detected by Northern blot analysis (see Fig. 3). Thus, it is doubtful that those signals are functional in vivo, at least in early embryos. All of the above demonstrate that SKh11 represents a full-length cDNA clone of p62.

**In Vitro Translation of p62**—The protein encoded by clone SKh11 has a calculated $M_r$ of 46,388. On SDS-gels, p62 has been estimated to have a $M_r$ of 62,000 (5). Thus, the molecular mass predicted from the sequence of SKh11 is 16 kDa less than the apparent molecular mass estimated from SDS-gels. Although phosphorylation may contribute to the anomalous migration of a given protein on SDS-gels (18), this 16-kDa discrepancy in molecular mass of p62 most likely results from the regions in the primary sequence that are extraordinarily rich in acidic residues. Indeed, highly acidic amino acid sequences can cause large anomalous migrations of proteins on SDS-gels. This has been inferred intuitively, as such proteins bind SDS with relatively low efficiency, and demonstrated directly by fusion studies using cloned CENP-B cDNAs (19). To confirm directly that the acidic character of p62 is responsible for this migration anomaly, the insert from clone SKh11 was transcribed and translated. When the T7 promoter in SKh11 is used to drive a coupled transcription-translation reaction with reticulocyte lysate, a T7 dependent polypeptide is produced that migrates on SDS gels at $M_r$ of 61,000 (Fig. 2, lane C). No similar product is produced when T3 polymerase, whose promoter lies downstream of the insert, is used as the negative control (Fig. 2, lane A). The positive translation control for this experiment is luciferase, having a $M_r$ of 61,000 (Fig. 2, lane C). Thus, these data show that the large molecular weight of p62 estimated from SDS-gels is most likely due to anomalous SDS binding, and support the conclusion that SKh11 is a full-length cDNA clone of p62.

**Northern and Southern Blot Analyses**—Northern blot analysis was performed to determine the size and expression of p62 mRNA during early development in the sea urchin embryo. High stringency hybridization between the insert of SKh11 and total RNA identified a 3.5-kilobase message in unfertilized eggs and in embryos at all stages of development investigated (Fig. 3). It is clear that the p62 message is present maternally in unfertilized eggs and throughout development up to the gastrula stage. Only one mRNA transcript was detected during early development in the sea urchin. The size of the message

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**FIG. 2.** In vitro transcription and translation of the insert from clone SKh11. One microgram of plasmid DNA was transcribed and translated in the presence of [$^{35}$S]methionine. The positions of molecular weight standards ($\times 10^3$) are noted on the left. Lane A, the negative control, shows the translation products resulting from transcription driven by the T3 promoter. Lane B shows translation of the insert in SKh11 driven by the T7 promoter. Note that a specific product is driven by the T3 promoter.

**FIG. 3.** Northern blot analysis of p62 transcripts in eggs and embryos of the sea urchin. Fifteen micrograms of total RNA from unfertilized sea urchin eggs and embryos at various stages of development were resolved by electrophoresis in a formaldehyde/agarose gel. The RNAs were transferred to nitrocellulose and hybridized in 50% formamide at 42°C with a DNA probe obtained from the full-length cDNA clone SKh11, prepared by random primer labeling of the insert from SKh11. Lanes 1–9, RNA from developmental stages as indicated above each lane.
corresponds to that of the insert of clone SKh11, indicating that the cloned cDNA corresponds to a full-length transcript.

To determine the copy number of the gene encoding p62 in the sea urchin genome, as well as to identify related genes, high molecular mass DNA from unfertilized sea urchin eggs was digested with EcoRI, KpnI, or HindIII (1–3), separated by electrophoresis, transferred to nitrocellulose, and probed with the insert from the full-length cDNA clone SKh11 under high stringency conditions.

Sequence Analysis of p62—Analysis of the amino acid composition reveals that the p62 polypeptide is rich in glutamyl (19.7%), aspartyl (13.9%), and lysyl (12.9%) residues. No cysteine is present in p62, indicating that p62 does not fold by intrachain disulfide bonds or form interchain disulfide bonds with other proteins. Based on amino acid charge distribution and hydrophobicity, the p62 polypeptide can be divided into three distinct domains (Fig. 5): an NH2-terminal acidic domain (residues 1–120, \( M_r = 13,457 \); isoelectric point (pI) = 4.21), called domain N, a middle, also acidic domain (residues 121–320, \( M_r = 22,684 \); pI = 3.79), called domain M, and the COOH-terminal basic domain (residues 321–411, \( M_r = 10,281 \); pI = 10.16), named domain C. Domain M can be further divided into five subdomains, named m1 to m5. There are three acidic (m1, m3, and m5) and two basic subdomains (m2 and m4) which alternate with each other and are either extremely rich in aspartyl and glutamyl residues or in lysyl residues. Subdomain m1 (pI = 3.25) consists of a stretch of 36 amino acids (residues 121–156) 67% of which are acidic (17 Glu, 7 Asp). Subdomain m2 (pI = 12.06) contains 25 amino acids (residues 157–181), of which 40% are basic (8 Lys, 2 Arg). Acidic residues are absent from m2. Subdomain m3 (pI = 3.04) is composed of 19 amino acids (residues 182–200), 95% of which are acidic (12 Glu, 6 Asp). Subdomain m4 (pI = 10.58) spans 45 amino acids (residues 201–245) and contains 29% basic residues (10 Lys, 2 Arg, 1 His). Subdomain m5 (pI = 2.77) is composed of 75 amino acids (residues 246–320), of which 81% are acidic (36 Glu, 25 Asp).

The entire polypeptide is relatively acidic with a pI of about 4.01. Two-dimensional gels and immunoblots of mitotic apparatus proteins confirm that p62 has a pI of about 4.0 (10). However, analysis of the data in Fig. 5 shows that the acidic and basic residues are clustered, thus producing distinct subdomain structure in p62 which may be functionally important. Highly acidic amino acid segments are characteristic of a number of nuclear and chromosomal proteins and are thought to play a role in chromatin binding (19) possibly through electrostatic interactions with basic histones. Moreover, in p62 subdomains m2 and m4 and domain C are basic. Several microtubule-associated proteins (MAPs) have basic microtubule binding domains, suggesting that these basic regions in p62 may represent one or more microtubule-binding domains.

When hydrophathy was analyzed by the Kyte-Doolittle algorithm (20), the results showed two characteristic regions in p62. Domain N is composed of a combination of both hydrophilic and hydrophobic amino acids. This suggests that domain N may adopt an internal position in the tertiary structure or, perhaps more likely, p62 interacts via hydrophobic interactions with one or more other polypeptides in vivo. By contrast, the majority of the remaining 321 residues, domains M and C, are charged and thus are highly hydrophilic. Finally, p62 does not contain any P-loop motifs (of the form GXXXGK(T/S/G)) for nucleotide binding. Thus, p62 is not predicted to have microtubule-based motor activity.

The p62 primary sequence was also analyzed against the PROSITE data bank for conserved protein motifs and potential post-translational modification sites. Surprisingly, a search for known calcium/calmodulin-dependent protein kinase phosphorylation sites failed to reveal any such sites in p62. However, certain regions in p62 have similarity to a base consensus sequence for this kinase, and one or more of these may represent potential calcium/calmodulin-dependent protein kinase phosphorylation sites in p62. The literature (21, 22) indicates that calcium/calmodulin-dependent protein kinase phosphorylation sites, while similar, vary greatly in actual sequence. One can distill the varying domains, however, to a minimal essential consensus composed of a basic residue followed by two to three residues and then the phosphorylated residue. When p62 is searched for this consensus, similar sequences are noted at six positions. These sites will be discussed in relation to the calcium/calmodulin-dependent phosphorylation of p62 in more
Amino acid sequence comparison of a segment of p62 with a segment of rat nucleolar protein B23. The deduced amino acid sequence of p62 (top) was aligned with the rat nucleolar protein B23 (bottom) using BESTFIT of the GCG package. Significant homology was detected in the portion of the sequence indicated here. Residues 96–124 of p62 show a high degree of homology with residues 96–124 of the rat nucleolar protein. The GCG software indicates identical residues with vertical lines between a given pair of residues, while conservative substitutions are indicated by two dots and similar residues are indicated by a single dot.

PROTEIN SEQUENCE SIMILARITY BETWEEN p62 AND KNOWN PROTEINS

GenBank™ Search—Protein sequence similarity between p62 and known proteins was analyzed by searching the data libraries of GenBank™ 79.0, SWISS-PROT 26.0, and PIR 38.0 as well as the yeast genome data base. No significant homologies between p62 and any known proteins were noted. However, p62 showed some degree of similarity to a superfamily of nucleolar phosphoproteins which includes nucleolar protein NO38 from Xenopus (23), nucleophosmin from chicken (24), rat protein B23 (25), and nucleolar phosphoprotein B23 from human (26, 27). These phosphoproteins are the major nucleolar proteins of growing eukaryotic cells. They have been found to associate with intranucleolar chromatin and preribosomal particles and bind to single-stranded nucleic acids (28). Although their function is unknown, these proteins are thought to play a role in pre-rRNA transcription and ribosome assembly (29).

Overall, p62 and the rat protein B23 (25) are 61% similar. The similarity, which is due mainly to the extensive stretches of glutamate and aspartate residues that occur in both proteins, is not significant. However, significant sequence homology is confined to a stretch of 29 amino acids from residues 96 to 124 (Fig. 6) in the N domain of p62 and the NH₂-terminal region of B23. A closer comparison of this region with other proteins identified exclusively 12 members from the nucleolar phosphoprotein superfamily, which are all 100% homologous in the region encompassing residues 105–115 of p62. However, no function has thus far been elucidated for this conserved region from rat B23 or other members of the family. p62 is clearly distinct from rat protein B23, as little sequence similarity is observed in the remainder of p62 and B23. When the p62 sequence was searched for consensus nuclear localization signals, none were found, an observation that is consistent with previous immunofluorescence data (10), which indicated that p62 interacts directly with chromatin prior to nuclear envelope reassembly.

Immunodetection of Phosphoserine, Phosphothreonine, and Phosphotyrosine in p62—As mentioned earlier, CNBr cleavage of in vitro phosphorylated p62 detected two major radioactive cleavage products via autoradiography, suggesting that, in vitro, p62 is phosphorylated on more than one residue by calcium/calmodulin-dependent protein kinase (5). To investigate this possibility, Western blot analysis of mitotic apparatus phosphoproteins not previously subjected to in vitro phosphorylation was performed using antibodies specific for phosphoserine, phosphothreonine, and phosphotyrosine residues. As shown in Fig. 7, all three antibodies react with p62, while other polypeptides on the blot react variably. This suggests that p62 contains at least one of each type of phosphoamino acid and is thus phosphorylated at multiple sites as suggested by CNBr cleavage studies. However, this result does not indicate whether any or all of these in vivo phosphoepitopes result from calcium/calcmodulin-dependent protein kinase activity.

Nucleus Immunolabeling—As revealed by homology searches, p62 showed some similarity to certain nuclear proteins and DNA-binding proteins. To study the possible interaction between p62 and chromatin/chromosomes, laser confocal microscopy was performed using affinity purified antibodies to p62 and antibodies to DNA. When nuclei were isolated from sea urchin eggs, processed for indirect immunofluorescence, and scanned by laser confocal microscopy, p62 was observed distributed throughout the nucleus, coincident with the decondensed chromatin characteristic of these cells, which have completed meiosis and are awaiting fertilization to activate the cell cycle (Fig. 8, a–c). By comparison, a similar analysis with germinal vesicles isolated from surf clam oocytes, which are arrested at prophase of meiosis I and have the bulk of their chromatin condensed and bound to the inner nuclear envelope, demonstrates that p62 resides throughout the nucleus, in association not only with the condensed chromosomes but also with other filamentous networks within the nucleoplasm (Fig. 8, d–f). These networks may represent decondensed chromatin, the transcription of which is required for oocyte maintenance. After stimulation of the completion of meiosis by KCl activation, these networks become less evident as the filamentous material progressively associates with the chromosomes during the final stages of chromatin condensation. Concurrently, p62 staining becomes restricted to the condensed chromatin as well (Fig. 8, g–i). These data provide direct evidence that p62 can be found in association with chromatin/chromosomes as suggested by the sequence analysis.

DISCUSSION

This study reports the sequence and characterization of p62, a mitotic apparatus-associated phosphoprotein important for mitotic progression in the sea urchin embryo. By SDS-PAGE
p62 has been estimated to have a relative molecular mass of 62 kDa (5), yet the relative molecular mass predicted from the cDNA sequence (Fig. 1) is 46 kDa. The large apparent mass on SDS-PAGE is due to the acidic properties of the protein, and this was demonstrated directly by in vitro translation of the cloned insert (Fig. 2). Although phosphorylation can affect the mobility of proteins on SDS gels (18), the major contributor to the migration anomaly noted here is the acidic nature of p62 (see Figs. 1 and 2) (19). The detection of phosphorylated CNBr-derived peptides by autoradiography, and the positive immunoreaction of p62 with anti-phosphoamino acid antibodies (Fig. 7), suggest that p62 is phosphorylated at multiple sites in vivo and in vitro. However, phoshopeptide mapping of p62 will be required to identify the number and position of these sites.

Previous studies (6, 8, 10) have shown that p62 associates with microtubule in the mitotic spindle. It is believed that microtubule-binding to associated proteins (MAPs) is mediated through electrostatic interactions between the acidic COOH terminus of β-tubulin and basic MAP domains (30, 31). For example, the microtubule binding domain shared by MAP 2, tau, and MAP 4 is characterized by three to four imperfect 18-amino acid repeats containing the characteristic tetramer sequence PGKG (32–36). By contrast MAP 1A and MAP 1B have a repeated binding motif of KKKE/EIV (37, 38). In addition, CLIP-170, a cytoplasmic linker protein that binds endocytic vesicles to microtubules, contains a conserved repeated motif, GKN/DSG, shared by rat DP-150, the Drosophila glued protein, and the yeast protein BIK1 (39). None of the above microtubule binding motifs was found in p62, and p62 shares no sequence homology, at the amino acid level, with other known MAPs. EMAP, a 77-kDa echinoderm MAP originally identified in eggs by cycles of pH- and temperature-dependent microtubule assembly and disassembly, has been reported in sea urchin, sand dollars, and starfish (40) to localize to interphase and mitotic microtubule arrays. This 77-kDa EMAP contains basic and slightly acidic regions in its deduced amino acid sequence but lacks the above-described characteristic microtubule-binding sequences (41). Basic and acidic charge distribution is typical of microtubule-binding proteins, and thus the basic region of EMAP may represent a novel microtubule binding domain. However, direct tests of this hypothesis, by, for example, constructing fusion proteins containing various EMAP domains, have not yet been reported. While a known microtubule-binding sequence in p62 similarly cannot be identified due to sequence homology alone, previous immunoelectron microscopy of isolated mitotic apparatuses (8) as well as immunofluorescence studies (6, 10) strongly suggest the association of p62 with microtubules in the mitotic spindle, perhaps either directly, through a basic, yet novel, microtubule-binding motif, or indirectly through other adapter proteins or MAPs.

Conversely, it has been reported that tubulin interacts with MAP-2 or cytoplasmic dynein at an EGEE sequence located in the acidic COOH terminus of α-tubulin (31, 42) (but see also Cleveland et al. (43)); an EGEE sequence (residues 147–150) is also present in the acidic subdomain, m1, of p62. A BLASTP search of the protein data base using this sequence detected many unrelated proteins. The significance of this sequence for non-tubulin proteins remains to be determined, but it may represent a motif for interaction of non-tubulin proteins with MAPs. In this regard, p62 could interact with certain MAPs through this element, and then the microtubule-binding ability of the MAP could mediate the association of p62 with microtubules.

p62 was originally identified as a substrate of a Ca\(^{2+}\)/calmodulin-dependent protein kinase (Cam kinase) which copurified with p62 in the sea urchin mitotic apparatus. Three classes of Cam kinases have been described thus far. Cam kinase I and Cam kinase III are specific to brain and certain other tissues, and each has a very narrow substrate specificity. By contrast, Cam kinase II is a multifunctional protein kinase...
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having broad tissue distribution and substrate specificity. p62 is likely phosphorylated by Cam kinase II based on the multifunctional property of the kinase and the fact that active Cam kinase II has been detected in isolated sea urchin mitotic spindles (7). One of the remarkable features of Cam kinase II phosphorylation site sequences is their variability. The enzyme recognizes the following motif, RXXS*TS* (asterisks indicate the phosphorylated residue) in both proteins and peptide substrates (22). Representative examples are synapsin I (44), tyrosine hydroxylase (45), and the autoprophosphorylation sites on the α and β subunits of Cam kinase II itself (46). However, different phosphorylation site sequences other than this consensus motif have been identified in many other proteins. For example, myelin basic protein has the phosphorylation site sequence RSKYLA*AST (47), ATP-citrate lyase contains R/KTAS*FSES R (48), and acetyl-CoA carboxylase has FIIGVS*EDN (49). In general, these sites, as well as the consensus motif, have a characteristic serine or threonine 2–6 residues following a basic residue. The primary sequence of p62 does not contain the consensus recognition sequence RXXS*TS* for Cam kinase II. However, considering the variability of the phosphorylation domains in CaM kinase II substrates, this is not surprising. In the p62 sequence, there are several S or T residues following one or more basic residues, such as 159KKGS, 209RPAPS, 226KDGT, 355KKTYS, and 369KSPS. Some of these sequences may serve as phosphorylation sites for Cam kinase II. Indeed, when p62 is phosphorylated in vitro, two CNBr peptides, referred to as pL and pS, are obtained, both of which are radiolabeled. Both PL and pS contain potential Cam kinase phosphorylation sites, 159KKGS in pL, 209RPAPS and 226KDGT in pS, which likely account for this observation. However, it should be noted that results from in vitro phosphorylation experiments may produce different phosphorylated residues than occurs in vivo. This question is currently being addressed by identifying and comparing the phosphoamino acid residues in p62 produced by in vivo versus in vitro phosphorylation reactions.

In addition to Cam kinase II, analysis of the p62 sequence revealed the presence of several potential phosphorylation sites for cAMP-dependent protein kinase, protein kinase C, casein kinase II, and tyrosine kinase. It is interesting to note the potential of cAMP-dependent phosphorylation of p62. cAMP-dependent phosphorylation of p62 in the isolated mitotic apparatus does not occur to any great extent (5), perhaps because the site is already phosphorylated when the mitotic apparatus is isolated. Of interest in this regard is a recent report (50) showing that cAMP-dependent protein kinase A is activated by maturation promoting factor and required for the transition from mitosis to interphase. The authors indicate that the cAMP-protein kinase A pathway is activated in the embryonic cell cycle, either directly or indirectly, by maturation promoting factor. These results suggest that the control of activation-inactivation cycles of the cAMP-protein kinase A pathway via maturation promoting factor may play a critical role in regulating transitions through M phase of the cell cycle. Recall this is the time in the cell cycle in which p62 is required, as antibodies to p62 arrest the cell cycle at the metaphase-to-anaphase transition when they are injected into dividing sea urchin embryos (6). Furthermore, previous work has shown that microinjection of the protein inhibitor of cAMP dependent protein kinase blocks the first division in sea urchin embryos prior to or during spindle formation (51).

p62 is a nuclear protein (10), is chromatin/chromosome-associated (Fig. 8), and is highly acidic (Fig. 5). Other chromatin-associated proteins, such as HMG-1 (52), also contain acidic domains. Indeed, a large class of nuclear proteins called A- proteins contain an extended region (or regions) enriched in acidic residues (19, 53). This class includes various proteins whose structures are not conserved and whose functions are not identical. It has been demonstrated that A- proteins exhibit significant binding to the core histones in vivo (54). This suggests that the acidic regions of p62 may serve to bind p62 to histones or to the basic domains in other chromosomal proteins. Considering the cell cycle-dependent nuclear localization of p62, this interaction is a likely candidate for the mechanism that targets p62 to the nucleus in late anaphase (10). It will be important to determine how p62 interacts with chromosomes/chromatin and what biological function this interaction serves in vivo. To this end, cloned p62 sequences are currently being used in in vitro expression experiments to obtain purified p62, and truncated clones are being used to identify the tubulin and chromatin binding domains of this polypeptide.

How might calcium- and calmodulin-dependent phosphorylation of p62 be involved in mitosis? In vivo, microtubules are sensitive to intracellular calcium concentrations, an effect mediated by calmodulin. For example, microinjection of fibroblasts with calcium-saturated calmodulin induces a rapid disruption of microtubules at the injection site (55, 56). Furthermore, microinjection of calcium chelators inhibits mitosis (57–59), again implicating calcium in this process. Finally, indirect immunofluorescence has localized calmodulin to the kinetochore microtubules of the mitotic apparatus (60, 61), and, in a related approach, fluorescently labeled calmodulin microinjected into cells binds to the kinetochore microtubules (62, 63). Taken together, these studies strongly implicate calcium and calmodulin in the controlled depolymerization of microtubules that is necessary for anaphase A. Consistent with this hypothesis is the observation that the calmodulin inhibitor, chlorpromazine, blocks mitosis in mammalian cells (64) and that antisense calmodulin RNAs under the control of the Zn2+-inducible metallothionein promoter cause a transient cell cycle arrest in mouse cells in the presence of Zn2+ (65). Therefore, calcium may act in concert with calmodulin to destabilize the kinetochore microtubules at the metaphase to anaphase transition. This in turn may permit chromosome movement to the poles along the now depolymerizing kinetochore microtubules, via a motor localized to the kinetochore (66–75). One obvious molecule that may be involved in the cascade of events that begins with calcium release and ends with the successful completion of mitosis is p62, which interacts with the microtubules of the mitotic apparatus (6, 9, 10), thus stabilizing them. When calcium is released to initiate anaphase, calcium-calmodulin dependent protein kinase II is stimulated to phosphorylate p62. We have previously shown that this kinase is a component of the isolated mitotic apparatus and that it can phosphorylate p62 in vitro (7). Mitotic apparatus microtubules with p62 phosphorylated are unstable (5) and therefore may be readily disassembled by the anaphase machinery that is undoubtedly localized to the kinetochores. This model provides the framework for the design of experiments to test a number of important hypotheses predicted by the model. Such experiments are currently in progress.

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