BIMA, a TPR-containing Protein Required for Mitosis, Localizes to the Spindle Pole Body in *Aspergillus nidulans*

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Abstract. The *Aspergillus nidulans bimA* gene is required for mitosis. Loss of function mutations in *bimA* cause cells to arrest growth with condensed chromatin and a short, metaphaselike mitotic spindle. *bimA* is a member of a gene family defined by a repeated motif called the Tetratrico Peptide Repeat (TPR), which is found in genes from bacteria, yeast and insects. Several yeast TPR genes are also required for mitosis, including *Saccharomyces cerevisiae* CDC27 and *Schizosaccharomyces pombe nuc2*$^+$, which appear to be functional homologs of *bimA*. We have developed antisera specific to the *bimA* protein (BIMA) and have characterized BIMA by western blot and immunocytochemical analyses. BIMA is heterogeneous in apparent molecular weight, consisting of a major 90-kD species and at least two minor species of ~105 kD. The results of BIMA localization by immunofluorescence microscopy depend on the level of BIMA expression. Overexpression of BIMA, which had no deleterious affect on growth or mitosis, resulted in localization of BIMA on or throughout most nuclei. Nuclear staining was granular, and overlapped but was not completely coincident with DNA staining by DAPI. In contrast, when expressed at normal levels, BIMA colocalized with the spindle pole body (SPB). BIMA localized to the SPB in a cell cycle independent manner. These results show that BIMA is either associated with or is a component of the SPB, and they suggest that BIMA functions at the spindle poles to promote the onset of anaphase.

MITOSIS is one of the most fundamental processes in eukaryotic cells. Accordingly, the structure and gross composition of the mitotic apparatus have been extensively studied. The role of spindle fibers and microtubule based motors in chromosome segregation are, in principle, understood (reviewed by McIntosh and Hering, 1991). Nevertheless, the mechanisms regulating spindle assembly and function and coordinating chromosome movements are largely unknown. Genetic investigations of mitosis have been undertaken with the hope of uncovering these mechanisms and identifying the molecules involved.

Morris (1976) isolated mutations in several *Aspergillus nidulans* genes that caused mitotic defects. Conditionally lethal mutations in one of these genes, *bimA*, caused cells to arrest growth with condensed chromatin and a short, metaphaselike mitotic spindle. Disruption of *bimA* by integrative transformation is lethal and phenocopies the original *bimA1* mutation, suggesting that the mutant phenotype is due to loss of *bimA* function (O'Donnell et al., 1991). The *bimA* sequence predicts a 90-kD protein containing 10 copies of a degenerate, 34 residue motif called the Tetratrico Peptide Repeat or TPR[1] (Sikorski et al., 1990; Hirano et al., 1990). TPR proteins are encoded by several other fungal genes required for mitosis (*Saccharomyces cerevisiae CDC16, CDC23, CDC27,* and *Schizosaccharomyces pombe nuc2*$^+$ and *cut9*$^+$) as well as fungal genes involved in RNA biogenesis (*S. cerevisiae SSN6, SKI3, PRP6,* and *STI1*) and mitochondrial protein import (*S. cerevisiae MAS70* and *Neurospora crassa MOM72*). Thus, the TPR motif itself is not specific for mitotic or nuclear functions. The identification of TPR genes in *Drosophila* and *Synechococcus* (a blue green bacterium) suggests that the TPR motif is of ancient evolutionary origin (Sikorski et al., 1991).

bimA is most highly related in structure and function to the TPR genes *CDC27* and *nuc2*$^+$. Mutations in all three genes result in similar, metaphaselike, mitotic blocks (Goebl and Yanagida, 1991). The TPRs of *bimA*, *CDC27*, *nuc2*$^+$ are positioned identically, with one TPR in the amino-terminus and nine tandem TPRs in the carboxy terminus (for example, see BIMA, Fig. 1). The sequences within TPR regions are ~50% identical in all three pairwise comparisons. Moreover, expression of *bimA* in yeast complements deletion of *CDC27*, and creation of a *cdc27* mutation that corresponds exactly to the original ts *nuc2-663* lesion creates a ts *cdc27* allele (Sikorski et al., 1991). These data strongly suggest that *bimA*, *nuc2*$^+$, and *CDC27* are functional homologs, and that they represent a central, evolutionarily conserved component or regulator of mitosis.

1. *Abbreviations used in this paper*: BIMA, *bimA* protein; PEM, 100 mM Pipes pH 6.9, 25 mM EGTA, 5 mM $MGSO_4$; PIB, protein isolation buffer; SPB, spindle pole body; TPR, tetratrico peptide repeat.

No specific biochemical function is known for any of the TPR proteins that are required for mitosis. Each TPR is predicted to form two, amphipathic helices, potentially involved in protein–protein interactions. Analyses of the TPR region of the nuc2$^+$ protein by circular dichroism and protease digestion support this prediction (Hirano et al., 1990), and Sikorski et al. (1991) demonstrated that the TPR proteins encoded by *CDC23* and *CDC27* are capable of forming homo- and hetero-dimers *in vivo*. Thus, physical interactions between TPR proteins may play a role in mitosis. There is also evidence that TPR proteins interact with a class of proteins related to the β subunit of transducin (Keleher et al., 1992; Williams et al., 1991; Goebl and Yanagida, 1991), a heterotrimeric G protein involved in signal transduction. We do not know how or if these interactions contribute to the function of mitotic TPR proteins.

Here we report the results of our characterization of the *bimA* protein (BIMA). We show that *bimA* encodes a 90-kD protein and several higher mol wt species that potentially represent posttranslationally modified forms of BIMA. The results of our immunocytochemical studies depend on the level of *bimA* expression. When overexpressed, BIMA colocalized with nuclei. The nuclear BIMA staining pattern was granular, and did not completely coincide with DNA staining by DAPI. When expressed at normal levels, BIMA localized to the spindle pole body (SPB). These results, together with those from mutational analyses, provide evidence that BIMA, and perhaps other mitotic TPR proteins, may play a role in spindle pole function during the transition from mitotic metaphase to anaphase.

Materials and Methods

Strains and Microbiological Techniques

A. nidulans strains used in this study were R153 (*wA2*; *pyroA4*), SWJ216 (*yA2*; *methB3*; *nimT23*), GR5 (*pyrG89*; *wA2*; *pyroA4*), TPM103.15 (GR5 transformed with pPM106), TPM200 (GR5 transformed with pPM104), TPM203 (GR5 transformed with pKO40). TPM103.15, TPM200, and TPM203 each contain a single copy of the indicated plasmid integrated by homologous recombination at the *bimA* locus (data not shown). Integration of pPM106 at the *bimA* locus of GR5 to make TPM103.15 preserved the wild-type *bimA* gene (data not shown). Integration of pPM104 at the *bimA* locus of GR5 to make TPM200 disrupted the resident *bimA* gene and placed the full-length *bimA* coding sequence under control of the *alcA* promoter. Thus, TPM200 is *bimA*$^-$ on glucose medium (which represses the *alcA* promoter), and is *bimA*$^+$ on ethanol medium (which induces the *alcA* promoter). Standard conditions were used for *Aspergillus* growth (Käfer, 1977), genetics (Pontecorvo et al., 1953), and transformation (Osmani et al., 1987). Standard molecular biological techniques were used for propagating and purifying plasmids (Sambrook et al., 1989).

Plasmid Constructions

Plasmids were constructed using standard cloning procedures (Sambrook et al., 1989) and all enzymes and reagents were used according to conditions specified by the supplier (New England Biolabs, Beverly, MA). The nucleotide position numbers referred to here correspond to those shown in Fig. 4 of O'Donnell et al. (1991). pPM132 was made by insertion of a 1619 bp HindIII *bimA* cDNA fragment (position 489 to 2108) into the HindIII site of pUR290 (Rüther and Muller-Hill, 1983; Sambrook et al., 1989). pPM118 was made by insertion of a 897 bp BamHI *bimA* gene fragment (position 1296 to 2193) into the BamHI site of pATH2 (Koerner et al., 1991). pPM128 was constructed in two steps. First, a 1951 bp XhoI to SalI *bimA* cDNA fragment (position 359 to 2300) from pRS314G-*bimA* (Sikorski et al., 1991) was inserted into the SalI site of pATH10 (Koerner et al., 1991) to yield plasmid pPM127. The XhoI site, which was inserted by oligonucleotide mutagenesis via PCR, resulted in addition of CTCGAGAACC immediately 5′ to the *bimA* ATG start codon. pPM127 was partially digested with BamHI to drop out the *bimA* BamHI fragment from position 1296 to 2300, yielding pPM128. Thus, pPM128 contains *bimA* sequences encoding BIMA amino acid residues 1 through 314 artificially fused to residues 612 through 648. To simplify Fig. 1, BIMA residues 612 through 648 are not shown as part of pPM128. pKO40 was made by inserting a ~3,400 bp EcoRV fragment from pKO9 (O'Donnell et al., 1991), containing the entire *bimA* genomic coding sequence plus 70 bp of 5′ untranslated leader, into the SmaI site of pAL3 (Waring et al., 1989). pPM104 was made by digestion of pKO40 with BamHI and religation, which removed *bimA* sequences from position 1296 to the pAL3 BamHI vector site. The BamHI fragment from pKO40 that contains *bimA* sequences from position 2300 to the pAL3 vector BamHI site was inserted into the BamHI site of pPM104 to yield pPM106.

Antibody Production and Purification

BIMA-containing fusion proteins were isolated as inclusion bodies from cultures of bacteria harboring pPM132, pPM118, or pPM128 by using the procedures described by Koerner et al. (1991) except that for pPM132 cultures, fusion protein expression was induced by 1 mM isopropyl β-D-galactopyranoside. BIMA-β-galactosidase from pPM132 cultures was purified by SDS-PAGE, electroeluted from gel slices, and used to immunize rabbits. Immunizations, serum collections, and serum processing were performed by Hazleton Research Products, Inc. (Denver, PA). Antibodies specific to different regions of BIMA were affinity purified using pPM128- and pPM118-derived BIMA-TRPE fusion proteins. Inclusion bodies containing predominantly BIMA-TPRE fusion proteins were solubilized in 10 M urea containing 1 mM PMSF and then dialyzed against PBS plus PMSF at 4°C to remove the urea. Protein derived from bacteria harboring pPM128 remained soluble in PBS after removal of urea, and was coupled to AFFI-GEL 15 by using procedures recommended by the supplier (Bio-Rad Laboratories, Cambridge, MA). AFFI-GEL-coupled pPM128 fusion protein was then used to purify BIMA-specific antibodies by using standard procedures (Harlow and Lane, 1988). The antibodies were incubated with the affinity column material overnight at 4°C with gentle shaking, and the unbound antibodies were removed and saved. The column was washed extensively with 10 mM Tris-HCl pH 7.5, 500 mM NaCl, and then the bound antibodies were eluted with 100 mM glycine pH 2.5. The eluted antibodies were neutralized with one-quarter volume 1 M Tris-HCl pH 8.0 and then frozen and stored at −80°C.

Protein derived from bacteria harboring pPM118 was insoluble after removal of urea, therefore, we used 0.1% SDS to solubilize pPM118 fusion protein and then bound the protein to strips of nitrocellulose. The membrane strips were washed free of SDS using PBS, blocked with 3% BSA, and then used to purify BIMA-specific antibodies by the same procedure used with pPM128 affinity columns. Several aliquots of whole serum were depleted of antibodies against both pPM128 and pPM118 fusion proteins by several passages over pPM128 columns and pPM118 membranes. The purified serum fractions used in this study gave absorbance values at 280 nm of 0.48 for ANTI-128 and 0.44 for ANTI-118.

Protein Preparation and Western Blot Analysis

Aspergillus strains were grown in appropriately supplemented minimal medium (Käfer, 1977) containing either 50 mM glucose, 200 mM ethanol, or 200 mM ethanol plus 0.04% fructose as carbon source (see below). For preparation of total protoplast protein (Fig. 2 *a*), minimal medium containing ethanol plus fructose was inoculated with 5×10^6 spores/ml and incubated at 37°C for 16 h. The resulting mycelial culture was converted to protoplasts using the procedure of Yelton et al. (1984), which yields protoplasts essentially free of cell wall debris. The isolated protoplasts were lysed by douncing in protein isolation buffer (PIB, see below), and the protoplast lysate was frozen and stored at −80°C. For preparation of total mycelial protein (Fig. 2 *b*), minimal medium containing glucose was inoculated with 5×10^6 spores/ml and incubated at 37°C for 12 h. The culture was harvested by filtration, washed with glucose-free medium, and then incubated in minimal medium containing ethanol at 37°C for 6 h. The cultures were harvested by filtration, washed with fresh medium, and then frozen in liquid nitrogen. The frozen tissue was lyophilized, ground to a fine powder using a mortar and pestle, and then was mixed with 4 vol of PIB and 1/2 volume glass beads on ice. To facilitate protein extraction, the mixture was vortexed three times for 30 s with 1 min intervals on ice between vortexings. Undisrupted cells and large cell fragments were removed by filtration through autoclaved miracloth (Calbiochem, La Jolla, CA) and the filtrate was frozen and stored at −80°C.

The Pierce BCA Protein Assay system (Pierce Chemical Co., Rockford, IL) was used to determine protein concentrations, and the relative protein concentration of different samples was confirmed by SDS-PAGE and Coomassie blue staining. PIB contains 50 mM Tris-HCl pH7.4, 300 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP-40, 0.2% Triton X-100, 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 0.1 mM NaF, 0.1 mM sodium vanadate, 1 mM DTT, 0.1 mg/ml Nα-p-Tosyl-L-Arginine methyl ester, 0.3 mg/ml benzamidine, 0.01 mg/ml Nα-p-Tosyl-L-Lysine chloromethyl ketone, 0.01 mg/ml soybean trypsin inhibitor, 0.01 mg/ml *N*-Tosyl-L-Phenylalanine chloromethyl ketone, 0.01 mg/ml pepstatin, 0.01 mg/ml leupeptin, 0.01 mg/ml aprotinin, 5 μg/ml antipain, 0.01 mg/ml chymostatin, 4 mM o-phenanthroline, and 1 mM diisopropyl fluorophosphate (all chemicals were from Sigma Chemical Co., St. Louis, MO).

SDS-PAGE and Western blotting were performed using standard procedures (Harlow and Lane, 1988). Blots were blocked by incubation for at least 1 h at room temperature in TBS containing 5% nonfat dry milk and 0.05% Tween-20. Primary and secondary antibodies were diluted in TBS containing 1% BSA, and antibody incubations with membranes were carried out at room temperature for at least 2 h. Membranes were washed using TBS containing 500 mM NaCl and 0.05% Tween-20. The secondary antibody was Goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) diluted 1:2,000.

Immunofluorescence Microscopy

For cytological studies, cells were grown on coverslips in stationary liquid cultures in petri plates. Spores adhered to the coverslips during germination, and then were processed on the coverslips as described below. For analysis of BIMA overexpression, spores were grown in minimal medium containing 200 mM ethanol as the sole carbon source. For analysis of wild-type strains in rich medium, synthetic complete medium was used (Käfer, 1977). Cells were fixed and washed according to Oakley et al. (1990). For cell wall removal, coverslips were incubated on 100 μl drops of 2% novozyme 234 (batch PPM 2934, Novo Industri A/S, Bagsvaerd, Denmark) in pembals (100 mM Pipes pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$, 1 M Sorbitol, 1% BSA) at 28°C for various times ranging from 15 to 90 min (see below). For cells grown in minimal ethanol medium, 1% novozyme in pembals was used. After novozyme digestion, the cells were washed in PEM (100 mM Pipes pH 6.9, 25 mM EGTA, 5 mM $MgSO_4$) and then extracted with 0.2% NP-40, 10% DMSO in PEM for 45 to 60 s at room temperature. The cells were rapidly washed free of extraction buffer using PEM and then were stored in TBS containing 0.1% BSA at 4°C for up to 2 d without noticeable loss of BIMA or MPM2 staining.

Primary and secondary antibodies were diluted into TBS containing 1% BSA for use in staining cells. Anti-BIMA antibodies were used at 1:100 or 1:250 and MPM2 antibody (from P. N. Rao University of Texas, MD Anderson Hospital and Tumor Institute, Houston, TX) was used at 1:800. Incubations were carried out at 4°C for 16 to 24 h. The affinity purified secondary antibodies, which were purchased from Jackson ImmunoResearch (West Grove, PA), were: [CY3]-labeled, goat anti-rabbit IgG (used at 1:500); [CY3]-labeled, donkey anti-mouse IgG (used at 1:500); [DTAF]-labeled, donkey anti-rabbit IgG (used at 1:250); and [DTAF]-labeled, donkey anti-mouse IgG (used at 1:250). The [DTAF]-anti-rabbit antibody was preadsorbed to mouse serum proteins, and the [CY3]- and [DTAF]-anti-mouse antibodies were preadsorbed to rabbit serum proteins (Jackson ImmunoResearch). Incubations with secondary antibodies were carried out at 28°C for 1 to 2 h. Double staining of BIMA and MPM2 was done using two sequences of antibody treatments: (*a*) anti-BIMA; [CY3]-anti-rabbit; MPM2; [DTAF]-anti-mouse, and (*b*) MPM2; [CY3]-anti-mouse; anti-BIMA; [DTAF]-anti-rabbit. anti-BIMA and MPM2 fluorescence colocalized using both procedures. No specific staining by [DTAF]-anti-mouse was detected when MPM2 was omitted from double staining experiments using sequence *a*, nor was specific staining by [DTAF]-anti-rabbit detected when anti-BIMA was omitted from double staining experiments using sequence *b*.

The extent of novozyme digestion was a crucial parameter in the immunocytochemical analysis of whole *A. nidulans* germlings. Insufficient digestion prevented antibody access to intracellular structures and overdigestion destroyed the structures being studied. The optimum extent of novozyme digestion depended on the structure being studied. For example, cytoplasmic microtubules were relatively sensitive to novozyme. They were best detected in samples digested for 15 min and were undetectable in samples digested for 30–40 min, under the conditions used in this study. In contrast, MPM2 staining of the SPB was negative in samples digested for 15 min, was optimum in samples digested from 40–50 min, and was faint but detectable in samples digested for 70–80 min. Reproducible staining of SPBs with anti-128 was observed only in samples digested for 50–70 min. Under these conditions, cytoplasmic and spindle microtubules were not preserved and DAPI staining of nuclei was diminished. For this reason, we could not show double staining with anti-BIMA and anti-α-tubulin antibodies to demonstrate that the BIMA-stained mitotic SPBs were at the poles of the mitotic spindle. Shorter digestion times resulted in increased background fluorescence and loss of SPB staining, and this staining pattern was the same whether anti-128, anti-118, or anti-BIMA-depleted serum was used. No condition of novozyme digestion was found that gave specific staining of wild-type cells using anti-118 or whole serum depleted of anti-BIMA activity.

Results

BIMA is Heterogeneous in Apparent Molecular Weight

To study BIMA, we raised rabbit antisera against a bacterially produced fusion protein containing part of BIMA fused to *Escherichia coli* β-galactosidase (pPM132, Fig. 1). Most of the BIMA sequences present in the immunogen are unique to BIMA and are not highly conserved in other known TPR proteins. We affinity purified anti-BIMA-specific activity from whole serum using two different fusion proteins, each containing part of the BIMA region used in the immunogen, fused to the *E. coli trpE* protein (pPM118 and pPM128, Fig. 1). These two affinity purified antisera, anti-118 and anti-128,

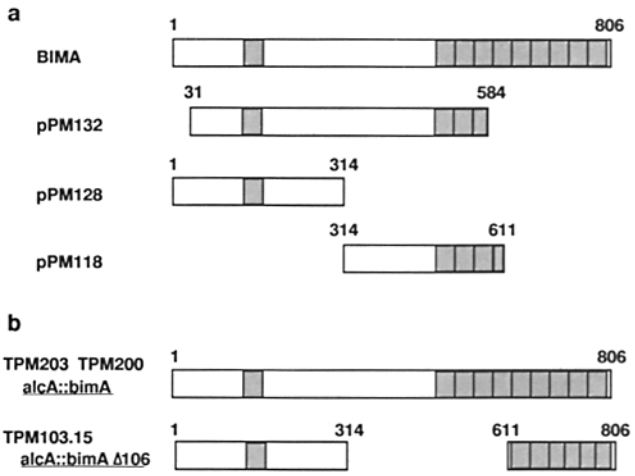

Figure 1. bimA-containing clones used for antisera production, immunoaffinity purification, and for BIMA overexpression studies. (*a*) Schematic representation of BIMA polypeptides used in antisera preparation, showing TPR domains as cross-hatched boxes and indicating the region of BIMA encoded by each clone. BIMA is the full-length polypeptide. pPM132, pPM128 and pPM118 are *bimA* gene fusion plasmids encoding the indicated portions of BIMA fused to either *E. coli lacZ* in pUR290 (pPM132) or to *E. coli trpE* in pATH2 (pPM128 and pPM118). Anti-BIMA antisera was raised in rabbits against pPM132 derived β-Galactosidase-BIMA fusion protein. Anti-BIMA activity was immunoaffinity purified using pPM128 and pPM118 derived TRPE-BIMA fusion proteins. (*b*) Schematic representation of BIMA sequences in *alcA::bimA* constructs used to make overexpression strains. TPM203 was constructed by transforming strain GR5 with pKO40 (*alcA::bimA*). TPM103.15 was constructed by transforming GR5 with pPM106 (*alcA::bimAΔ106*). TPM200 was constructed by transforming GR5 with pPM104 (see Materials and Methods). TPM200 contains *alcA::bimA* as its only functional *bimA* gene (the resident *bimA* locus is disrupted after amino acid residue 314), whereas TPM203 AND TPM103.15 also contain a wild-type *bimA* gene. The transforming plasmids integrated at the *bimA* locus in all three transformants.

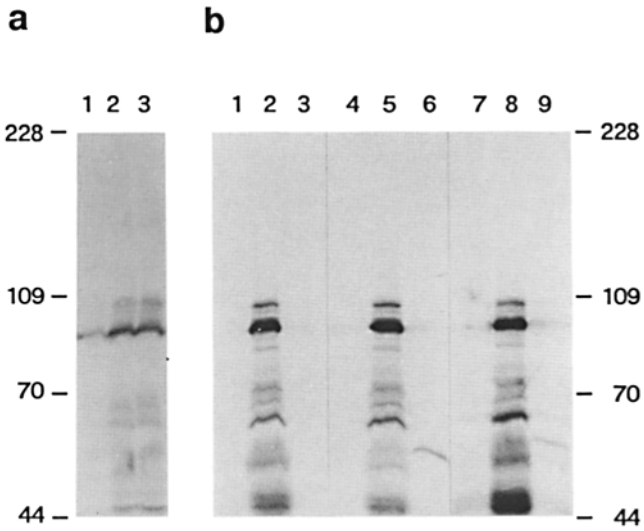

Figure 2. Characterization of BIMA by western blot analysis. (*a*) Total protein was isolated from protoplasts of several *A. nidulans* strains, which were prepared as described in the Materials and Methods. Equal amounts (13 μg) of protein were subjected to SDS-PAGE and transferred to nitrocellulose, and the blot was probed with a mixture of ANTI-118 (diluted 1:1,000) and ANTI-128 (diluted 1:1,000) affinity purified antisera. Lane *1*, strain R153 (*bimA* wild type control); lane *2*, strain TPM203 (*bimA, alcA::bimA*); lane *3*, strain TPM200 (*alcA::bimA*). (*b*) Total protein was isolated from mycelial cultures of several *A. nidulans* strains, as described in the Materials and Methods. Equal amounts (30 μg) of protein were subjected to SDS-PAGE and transferred to nitrocellulose, and the blots were probed with affinity purified ANTI-118 antisera diluted 1:1,000 (lanes *1–3*), ANTI-128 antisera diluted 1:1,000 (lanes *4–6*), or a mixture of ANTI-118 plus ANTI-128 both diluted to 1:1,000 (lanes *7–9*). Lanes *1*, *4*, and *7*, strain R153; lanes *2*, *5*, and *8*, strain TPM203; lanes *3*, *6*, and *9*, strain TPM103.15 (*bimA, alcA::bimAΔ106*).

each bound to its respective purifying antigen on western blots but did not cross-react with the alternative antigen (data not shown). Thus, anti-118 and anti-128 bind specifically to BIMA, but recognize different, nonoverlapping BIMA epitopes.

To facilitate the identification of BIMA in western blot and immunocytology experiments, we constructed *Aspergillus* strains containing the *bimA* coding sequence fused to the *A. nidulans alcA* promoter (*alcA::bimA*, Fig. 1 *b*). Transcription of *alcA* is repressed in medium containing glucose as carbon source and is highly induced in medium containing ethanol as carbon source (Lockington et al., 1985). Therefore, growth of *alcA::bimA*-containing strains in ethanol medium induces overexpression of BIMA. TPM203 contains a wild type *bimA* gene and one copy of *alcA::bimA*. TPM103.15 contains a wild-type copy of *bimA* and one copy of *alcA* fused to an internally truncated *bimA* gene (*alcA::bimAΔ106*). In TPM200, the resident *bimA* gene is deleted after amino acid residue 314 and the only functional copy of *bimA* is *alcA::bimA* (see Materials and Methods). All *alcA::bimA*-containing strains grow normally on ethanol medium, demonstrating that induction of *alcA::bimA* and hence overproduction of full-length or internally-truncated BIMA does not inhibit growth.

For Western blot analysis, we isolated protein from wild-type and several BIMA overexpressing strains grown in medium that induces *alcA::bimA*. Fig. 2 *a* shows that a mixture of anti-118 and anti-128 bound to several distinct protein species, including a major band at 90 kD and minor bands from 100 to 105 kD. All of these proteins accumulated to higher levels in strains TPM203 and TPM200 that were induced to overexpress full-length BIMA (lanes *2* and *3*). Fig. 2 *b* shows that both sera, when used individually, bound to the 90 kD and higher mol wt proteins (compare anti-118, lane *2* to anti-128, lane *5*). In protein extracts of TPM103.15 cells expressing full-length and truncated BIMA, ANTI-128 serum bound to the 90-kD band and to a novel protein of the appropriate mol wt (Fig. 2 *b*, lane *6*). anti-118 serum did not detect truncated BIMA (Fig. 2 *b*, lane *3*) because the truncation removed all the anti-118 epitopes (see Fig. 1). Anti-118 and anti-128 sera combined gave a stronger signal than either serum alone at the same antibody concentration (compare lanes *7*, *8*, and *9* to lanes *1–6*), confirming that both sera recognize the same proteins. Whole serum depleted for anti-BIMA activity did not detect any of the above proteins in wild type or in BIMA overexpressing strains (data not shown). The lower mol wt proteins shown in Fig. 2 are presumably due to proteolysis of BIMA because they are overproduced in BIMA overexpressing strains. These data demonstrated that both antisera specifically recognize only BIMA in *Aspergillus* extracts.

The major product of in vitro transcription and translation of *bimA* cDNA migrates as a 90-kD protein on SDS gels (data not shown), which agrees with the predicted mol wt of BIMA (89.7 kD, O'Donnell et al., 1991). Numerous experiments using different culture conditions and/or different protein extraction conditions gave results similar to those shown in Fig. 2. The 90-kD protein is, therefore, a primary *bimA* translation product and not a degradation product of the higher mol wt forms. Thus, BIMA is heterogenous in apparent mol wt, and consists of a major 90-kD form and minor forms from 100 to 105 kD, which potentially represent post-translationally modified forms of the 90-kD BIMA polypeptide.

Localization of BIMA to Nuclear Regions

To determine the appropriate conditions for using our BIMA-specific antisera in immunolocalization experiments, we initially examined strains overexpressing BIMA. We inoculated dormant, uninucleate spores into stationary liquid cultures and allowed them to germinate on coverslips for various times. During germination, each spore swells uniformly, adheres to the coverslip, and then extends a germ tube. Closed mitotic divisions occur during spore swelling and germ tube extension, and the nuclei migrate down the germ tube (Morris and Enos, 1992).

Fig. 3 shows that overexpression of BIMA resulted in BIMA-specific staining on or throughout most nuclei. The nuclear staining pattern was granular, and overlapped but was not always identical to the pattern of DNA staining by DAPI. Most but not all nuclei of overexpressing strains stained brightly with anti-BIMA sera (compare Fig. 3, *a* and *b*), and some nuclei showed weak staining (see arrow in Fig. 3, *a* and *b*). The exact percentage of nuclei that stained with anti-BIMA sera varied from experiment to experiment, presumably for technical reasons.

Anti-128 (Fig. 3, *c* and *d*) and anti-118 (Fig. 3, *e* and *f*)

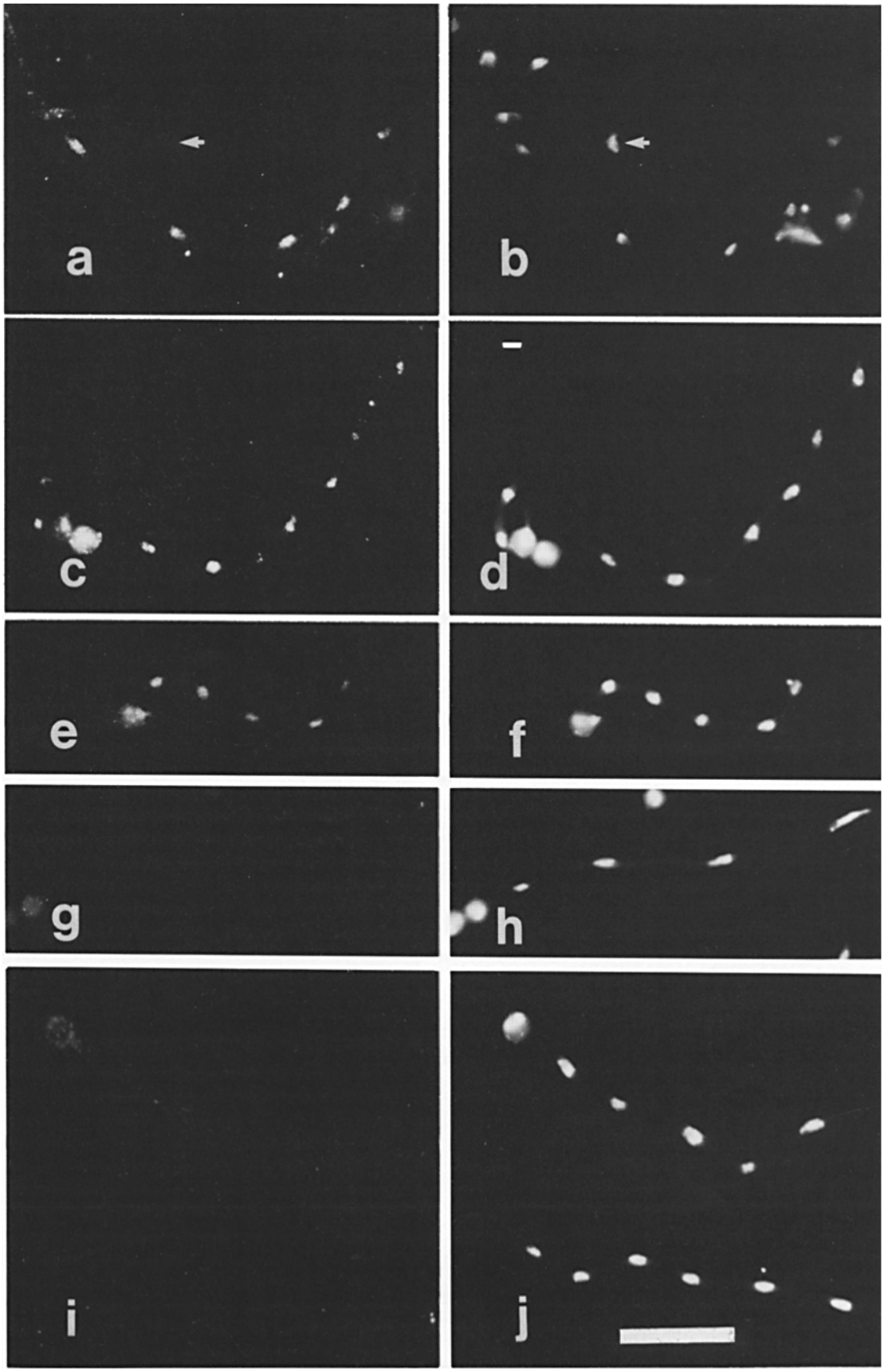

Figure 3. In situ localization of BIMA in overexpressing strains. Photomicrographs of cells that were grown in minimal medium with ethanol as the sole carbon source. Cells were fixed and prepared for immunocytology as described in Materials and Methods, and then stained with anti-BIMA or control antisera to localize BIMA. *a*, *c*, *e*, *g*, and *i* show anti-BIMA or control immunofluorescence and *b*, *d*, *f*, *h*, and *j* show DAPI fluorescence. (*a* and *b*) TPM203 cells stained with a mixture of ANTI-118 plus ANTI-128 sera each diluted 1:250; (*c* and *d*) TPM203 cells stained with ANTI128 serum diluted 1:100; (*e* and *f*) TPM203 cells stained with ANTI-118 serum diluted 1:100; (*g* and *h*) R153 cells stained with a mixture of ANTI-118 and ANTI-128 sera each diluted 1:100; (*i* and *j*) TPM203 cells stained with whole serum depleted of anti-BIMA activity diluted 1:100. The arrows in *a* and *b* show an example of a nucleus that stains weakly with anti-BIMA antisera. Bar, 20 μm.

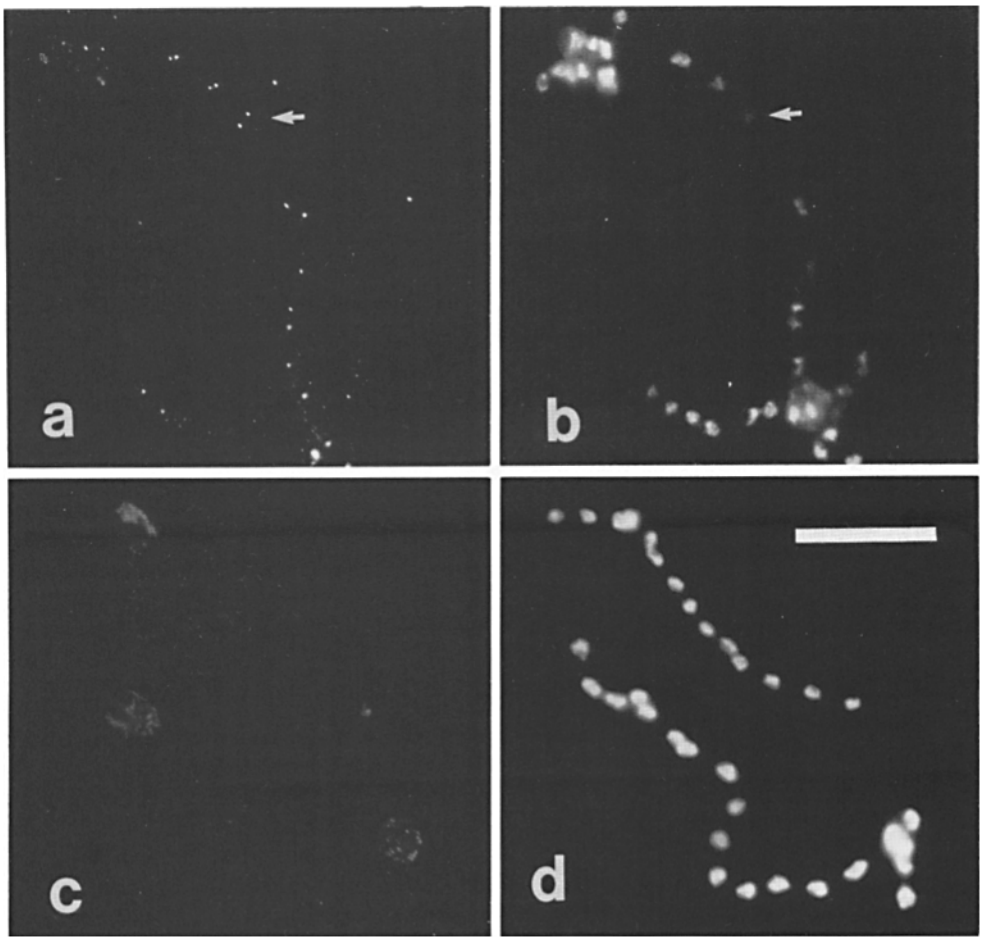

Figure 4. In situ localization of BIMA in wild type cells. Photomicrographs of R153 cells grown in rich medium, processed for immunocytology as described in Materials and Methods, and then stained with ANTI-128 serum (diluted 1:250) or whole serum depleted of anti-BIMA activity (diluted 1:100) and with DAPI to stain nuclei. (*a*) Anti-BIMA fluorescence; (*c*) fluorescence from anti-BIMA depleted serum; (*b* and *d*) DAPI fluorescence. Arrows in *a* and *b* show an example of nucleus that stains with two BIMA dots. Bar, 20 μm.

yielded similar results when used individually, and the combined antisera stained nuclei more strongly than either individual serum at the same antibody concentration. Nuclear localization was dependent on overexpression of full-length BIMA. Significant nuclear staining was not detected in parallel experiments with a wild-type strain (Fig. 3, *g* and *h*, also see below) or with TPM103.15, a strain induced to overexpress internally truncated BIMA (data not shown). Serum depleted of anti-BIMA activity did not stain nuclei of overexpressing strains (Fig. 3, *i* and *j*), confirming that this pattern is BIMA-specific.

Although general nuclear BIMA staining was not detectable in wild-type strains grown in ethanol medium, some dotlike staining was evident (Fig. 3 *g*). Further investigation of wild-type strains grown in rich, glucose-containing medium failed to detect the nuclear staining pattern seen in overexpressing strains, however, a striking dotted pattern was revealed (Fig. 4). Most of the bright dots corresponded to the positions of nuclei. The majority of cells had one BIMA-dot per nucleus, although a small percentage of cells had two BIMA-dots per nucleus (see arrow in Fig. 4, *a* and *b*). In numerous experiments, anti-BIMA serum stained nuclear-associated dots in virtually all cells. The nuclear dots were detected with anti-128 serum but not with anti-118 serum. Whole serum depleted of anti-BIMA activity did not stain the nuclear dots (Fig. 4, *c* and *d*), confirming that these dots are BIMA-specific.

We observed a variable number of faint, non-nuclear dots of anti-BIMA fluorescence in most experiments. Similar dots were detected using serum depleted of anti-BIMA activity, and the number and position of these dots varied from experiment to experiment and from cell to cell. These weaker staining, non-nuclear dots presumably represent nonspecific antibody binding because they were not more numerous or more intense in cells overexpressing BIMA (compare Fig. 3, *a*, *c*, and *e* with Fig. 4 *a*).

Localization of BIMA to the Spindle Pole Body

The BIMA-specific, nuclear-dot pattern is reminiscent of the results obtained in immunocytochemical studies using the monoclonal antibody MPM2 (Engle et al., 1988) and anti-γ-tubulin antisera (Oakley et al., 1990). Both of these antibodies stain the SPB of *A. nidulans*. In addition, 4% of nuclei in asynchronous cultures were associated with two BIMA dots, consistent with the expected frequency of mitotic nuclei which are associated with two SPBs (Bergen and Morris, 1983; Engle et al., 1988). Therefore, we investigated the possibility that BIMA localizes to the SPB by staining mitotic cells from a synchronous culture with anti-BIMA and with MPM2 antibodies.

To obtain a culture enriched for mitotic cells, we used SWJ216, an *A. nidulans* strain containing the temperature sensitive *nimT23* allele. *nimT* is homologous to *S. pombe* *cdc25*$^{+}$, which encodes the tyrosine phosphatase that dephosphorylates p34^{cdc2} to promote entry into M phase (O'Con-

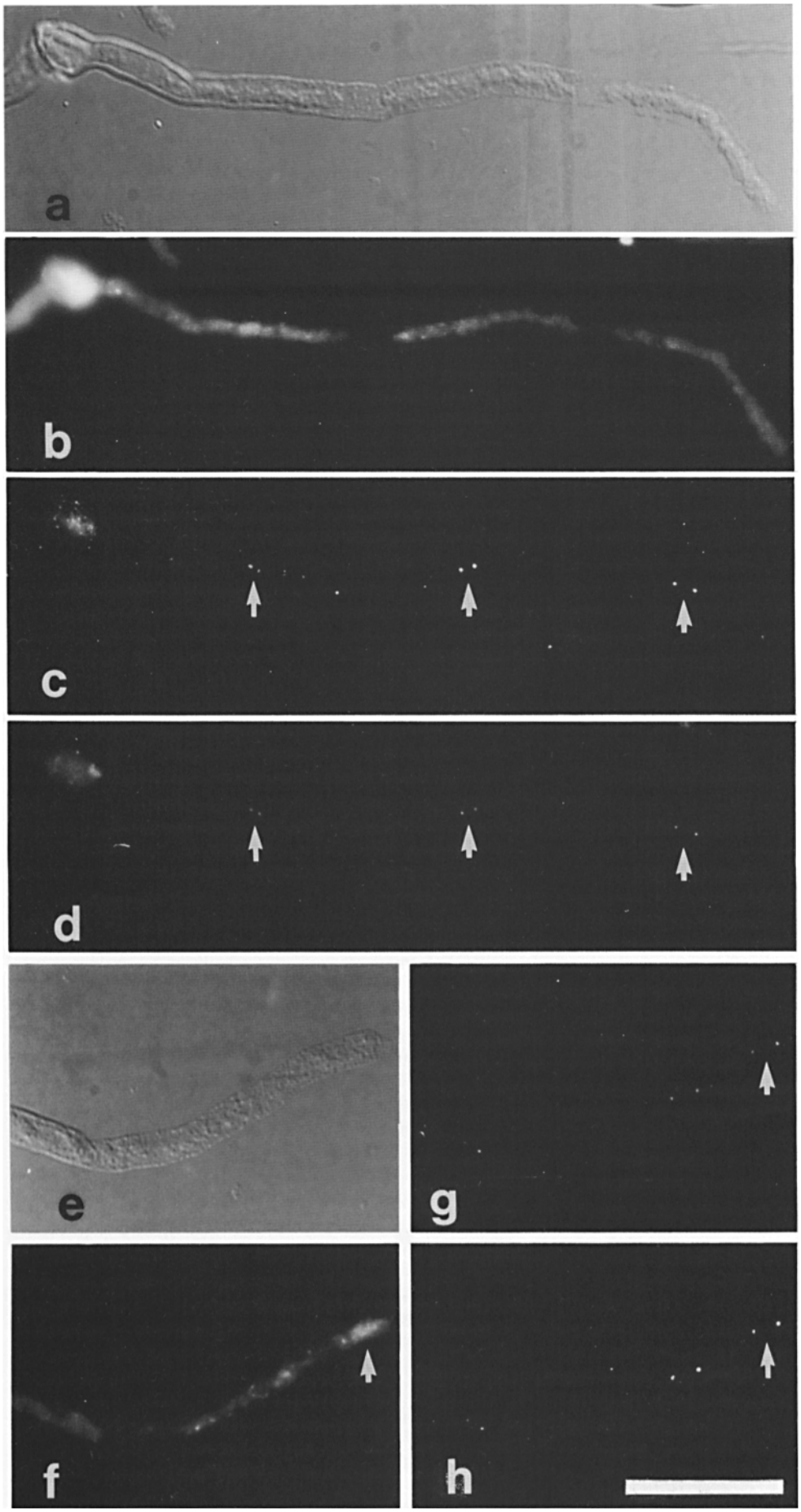

Figure 5. Localization of BIMA to the spindle pole body. Photomicrographs of synchronized SWJ216 cells grown as described below and processed for immunocytology as described in Materials and Methods. The cells were stained with ANTI-128 diluted 1:250 to detect BIMA, with MPM2 diluted 1:800 to stain the spindle pole bodies, and with DAPI to stain nuclei. SWJ216 spores were germinated at permissive temperature (25°C) overnight, until most cells contained two to four nuclei. Cultures were then shifted to 44°C for 3 h to arrest nuclear division at the G_2/M border. To release the cells into mitosis, the cultures were removed from 44°C, placed at room temperature, and diluted with an equal volume of 25°C medium. Samples were harvested and fixed at 4 min intervals. Samples harvested 8 min after release consisted of 70% mitotic cells as determined by α-tubulin immunofluorescence, therefore, 8 min samples were chosen for analysis with anti-BIMA and MPM2 antibodies. (*a* and *e*), Nomarski image; (*b* and *f*), DAPI fluorescence; (*c* and *g*), anti-BIMA fluorescence; (*d* and *h*), MPM2 fluorescence. Arrows in *c* and *d* show examples of duplicated spindle pole bodies that stain with both anti-BIMA and with MPM2 antibodies. Arrows in *f–h* show clearly that BIMA, MPM2, and DAPI fluorescence colocalize. Bar, 20 μm.

nell et al., 1992; Dunphy and Kumagai, 1991; Gautier et al., 1991). Incubation of SWJ216 at 44°C resulted in synchronization of cells at the G2 to M boundary, and shifting down to 28°C caused a rapid appearance of mitotic cells as judged by DAPI staining and anti-α-tubulin immunofluorescence (data not shown, also see Osmani et al., 1991). Samples of the released culture that contained mostly mitotic cells (greater than 70% with condensed chromatin and bipolar mitotic spindles) were chosen for staining with anti-BIMA, with MPM2, or with both.

Anti-BIMA and MPM2 antibodies both stained 2 dots on most nuclei. In doubly stained samples, the MPM2 antigens and BIMA colocalized (Fig. 5). Under the conditions necessary to obtain double staining with ANTI-BIMA and MPM2 (see Materials and Methods), the mitotic spindle was not preserved and DAPI staining of nuclei was greatly diminished. For example, Fig. 5, *a–d*, shows a cell positive for both BIMA and MPM2 SPBs but with almost no nuclear DAPI fluorescence above the background cytoplasmic fluorescence. The BIMA and MPM2 dots corresponded to the position of nuclei for all cells in which the nuclear morphology as revealed by DAPI fluorescence was preserved (for example, see arrows in Fig. 5, *f–h*). The apparent difference in distance between the spindle poles in the mitotic nuclei in Fig. 5, *c* and *d*, is due to different orientations of the spindles relative to the focal plane and is commonly seen in mitotic *Aspergillus* cells (unpublished observations). These results demonstrated that BIMA, when expressed at normal level, localized to the SPB.

Discussion

The *A. nidulans bimA* gene is one of several fungal TPR genes required for the completion of mitosis. The *S. pombe nuc2*$^+$ and *S. cerevisiae CDC27* TPR genes appear to be functional homologs of *bimA*, suggesting that *bimA* represents a fundamental, evolutionarily conserved mitotic component. We have developed two immunoaffinity purified antisera specific for BIMA in order to study its structure and subcellular localization in hope of gaining new insight into BIMA function.

Our results demonstrated that BIMA is a 90-kD protein and they suggest that some BIMA polypeptides undergo post-translational modification which significantly increases their apparent mol wt. An internally deleted form of BIMA exhibited no such heterogeneity in relative mobility on SDS-PAGE (see Fig. 2 *b*, lane *6*), suggesting that the deleted region is required for these modifications, and is potentially the region of BIMA that is modified. In this regard it is worth noting that this region of BIMA contains several consensus recognition sites for serine, threonine and tyrosine kinases and one site for N-linked glycosylation.

BIMA localized to the SPB in wild-type cells and localized on or throughout most nuclei in cells overexpressing BIMA. SPB localization was detectable in essentially all cells of asynchronous cultures, arguing against cell cycle regulation of BIMA localization. It is possible that the different BIMA isoforms detected on western blots localize to different subcellular locations in situ. Because both of our antisera recognize the same set of BIMA proteins (see Fig. 2 *b*) we cannot rule out the possibility that some BIMA species localize to the SPB while others localize throughout the nucleus.

The two patterns of BIMA localization are similar to those reported for localization of the *S. cerevisiae* SPB protein encoded by *KAR1* (Vallen et al., 1992). Moderate expression of certain *KAR1* fusion proteins resulted in SPB localization whereas high expression resulted in a general nuclear staining as well as SPB staining. As suggested for *KAR1*, overexpression of BIMA may saturate its binding site(s) at the SPB causing excess BIMA to accumulate in the nucleus. Alternatively, the nuclear and SPB localizations may both represent biologically significant locations for BIMA.

The functional and structural similarities of *bimA* and *nuc2*$^+$ suggest that their gene products may reside in similar subcellular locations. The *nuc2*$^+$ protein (NUC2) cofractionates with nuclei (Hirano et al., 1988) and localizes to the nucleus (Goebl and Yanagida, 1991). Those data, and the fact that NUC2 is insoluble in 2M NaCl, 2% NP-40, and 25 mM lithium iodo-salicylate, suggested that NUC2 may be a component of the nuclear scaffold (Hirano et al., 1988). Those results are also consistent with the hypothesis that NUC2 localizes to the SPB because, like NUC2, BIMA shows nuclear localization when it is overexpressed and the *S. pombe* SPB is likely to cofractionate with nuclei and have solubility properties similar to NUC2.

The fact that NUC2 was not detected at the SPB in *S. pombe* could have been because of technical reasons. Although both of our purified serum fractions gave good signals on Western blots of wild-type protein and good immunofluorescence in BIMA overexpressing strains, only ANTI-128 stained SPBs in wild-type strains. ANTI-118, which binds to internal BIMA sequences (Fig. 1), gave no discernable staining pattern above background when used on wild-type cells (data not shown). The ANTI-118 epitopes are evidently inaccessible when BIMA is at the SPB, and it is possible that the epitopes recognized by the NUC2 antibodies are similarly occluded. Rout and Kilmartin (1990) also reported that several SPB epitopes were undetectable in whole cells using formaldehyde fixation procedures and that some epitopes were more accessible to antibodies after chemical extraction removed microtubules and other SPB components. Indeed, our best staining of BIMA at the SPB is obtained under conditions in which cytoplasmic and spindle microtubule staining is not preserved (unpublished observations), suggesting that microtubules at the SPB could mask BIMA staining.

The phenotype of *bimA* mutations together with localization of BIMA to the SPB suggests that BIMA may play a role in SPB function during mitosis. If so, then *bimA* functions differently from most other genes thought to facilitate SPB function. For example, mutations in the *S. cerevisiae* genes *CDC31* (Byers, 1981), *MPS1* and *MPS2* (Winey et al., 1991), *NDC1* (Thomas and Botstein, 1986) and *KAR1* (Rose and Fink, 1987) all affect mitosis by perturbing SPB duplication. Inactivation of *A. nidulans mipA*, which encodes the SPB component γ-tubulin, disrupts mitosis by preventing spindle microtubule assembly (Oakley et al., 1990). One gene somewhat similar to *bimA* in mutant phenotype and protein localization is *KAR3*, which encodes a kinesinlike protein required for karyogamy in *S. cerevisiae* (Meluh and Rose, 1990). *KAR3* protein localizes to cytoplasmic microtubules and the SPB, and inactivation of *KAR3* causes 40% of mutant cells to arrest with duplicated SPBs and a short mitotic spindle. For *KAR3*, localization to the SPB is apparently required

only for karyogamy because some mutations in *KAR1* that abolish karyogamy and SPB localization of the *KAR3* protein do not affect mitosis (Vallen et al., 1992).

It seems likely that one aspect of BIMA function at the SPB will involve protein-protein interactions. Based on the results of biochemical and theoretical analyses of the NUC2 TPR domain, Hirano et al. (1990) proposed that each repeat associates with other repeats through hydrophobic interactions. The TPR proteins encoded by *S. cerevisiae CDC23* and *CDC27* form homo- and heterodimers in vivo (Sikorski et al., 1991), and they are coimmunoprecipitated by antibodies against the *CDC23* protein (J. Lamb and P. Hieter, personal communication). Although the TPR domains have not been proven to be responsible for the *CDC23/CDC27* interactions, these results support the idea that TPR-containing polypeptides physically interact. It is possible that BIMA may interact with other TPR proteins as part of BIMA's function at the SPB. It follows that the BIMA TPR domain itself may specify localization of BIMA to the SPB via similar interactions.

In addition to interacting with other TPR genes, genetic studies in *S. cerevisiae* indicate that many TPR genes interact with members of the β-transducin gene family. Examples include *PRP6/PRP4* (Dalrymple et al., 1989), *SKI3/MAK11* (Icho and Wickner, 1988), *SSN6/TUP1* (Williams et al., 1991), and *CDC20* with both *CDC23* and *CDC16* (D. Burke, personal communication). The best understood example is *SSN6/TUP1*, where biochemical and genetic experiments show that their gene products form a complex that acts as a general transcriptional repressor (Williams et al., 1991; Keleher et al., 1992). The fact that *cdc20* and *bimA* mutants have similar phenotypes (Sethi et al., 1991; O'Donnell et al., 1991) is consistent with a role for β-transducin-related proteins in mitosis. In fact, G-protein mediated signal transduction could provide a mechanism for propagating a signal between BIMA at the SPB and other parts of the mitotic apparatus, for example, the kinetochore.

Another way in which loss of BIMA function at the poles could be transmitted to the rest of the mitotic apparatus is through the activity of a diffusible regulatory protein such as $p34^{cdc2}$. The SPB is the site of localization of $p34^{cdc2}$ and cyclin B at metaphase in *S. pombe* (Alfa et al., 1990). *bimA* mutations could alter interactions between these regulators and the SPB by, for example, perturbing the process of cyclin B destruction. Degradation of cyclin B coincides with the onset of anaphase A, and inactivation of the $p34^{cdc2}$ kinase via turnover of cyclin B is necessary for the metaphase to anaphase transition (Murray et al., 1989; Forsburg and Nurse, 1991). Therefore, it is possible that mutations in *bimA* directly or indirectly prevent inactivation of $p34^{cdc2}$, resulting in a failure to exit metaphase.

It is also possible that *bimA* mutations disrupt events at the poles that are directly involved in the metaphase to anaphase transition. An interesting example of such an event is kinetochore microtubule disassembly at the spindle poles during metaphase in mammalian cells (Mitchison et al., 1986; Mitchison, 1989; Mitchison and Sawin, 1990) and during both metaphase and anaphase-A in Newt lung cells (Mitchison and Salmon, 1992). Spindle microtubule disassembly is important for mitosis in *Aspergillus* because *benA33*, a mutation in the *A. nidulans* β-tubulin gene, causes formation of hyperstable microtubules that block mitosis at metaphase (Oakley and Morris, 1981). Mutations in *mipA*, the gene encoding the SPB component γ-tubulin, suppress the lethality and microtubule hyperstability of *benA33* (Weil et al., 1986; Oakley and Oakley, 1989; Oakley et al., 1990), demonstrating that microtubule destabilization at the spindle poles is necessary for transition from metaphase to anaphase in *Aspergillus*. The phenotype of *bimA* mutations and localization of BIMA to the SPBs suggests that BIMA could play an important role in the dynamic interactions between kinetochore microtubules and spindle poles during mitosis.

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