Nucleus-Specific Importin Alpha Proteins and Nucleoporins Regulate Protein Import and Nuclear Division in the Binucleate Tetrahymena thermophila

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The ciliate Tetrahymena thermophila, having both germ line micronuclei and somatic macronuclei, must possess a specialized nucleocytoplasmic transport system to import proteins into the correct nucleus. To understand how Tetrahymena can target proteins to distinct nuclei, we first characterized FG repeat-containing nucleoporins and found that micro- and macronuclei utilize unique subsets of these proteins. This finding implicates these proteins in the differential permeability of the two nuclei and implies that nuclear pores with discrete specificities are assembled within a single cell. To identify the import machineries that interact with these different pores, we characterized the large families of karyopherin homologs encoded within the genome. Localization studies of 13 putative importin (imp) ω- and 11 imp β-like proteins revealed that imp α-like proteins are nucleus specific—nine localized to the germ line micronucleus—but that most imp β-like proteins localized to both types of nuclei. These data suggest that micronuclear-specific proteins are transported by specific imp α adapters. The different imp α proteins exhibit substantial sequence divergence and do not appear to be simply redundant in function. Disruption of the IMA10 gene encoding an imp α-like protein that accumulates in dividing micronuclei results in nuclear division defects and lethality. Thus, nucleus-specific protein import and nuclear function in Tetrahymena are regulated by diverse, specialized karyopherins.

The regulated transport of macromolecules into and out of the nucleus controls many cellular processes. The import of proteins containing nuclear localization signals (NLSs) is facilitated by karyopherin proteins, also known as importins. Karyopherins shuttle their NLS-containing cargo through nuclear pore complexes (NPCs), protein channels that permeate the nuclear membrane. Two classes of karyopherins, imp α and β proteins, collaborate to orchestrate specific nucleocytoplasmic trafficking (reviewed in reference 13). To transport proteins containing a classical NLS (cNLS), imp α binds to the cargo’s cNLS and associates with imp β in a ternary complex that docks at an NPC (reviewed in reference 23). The karyopherin-cargo complex is directed through the NPC, primarily through interactions of imp β with NPC proteins (6, 7, 26). Nuclear proteins may, alternatively, contain nonclassical NLSs that mediate direct association with imp β proteins without the imp α adapter.

Eukaryotic cells typically encode multiple karyopherins. In yeast and humans, which contain at least 14 and 20 karyopherins, respectively, most are members of the imp β family. Yeast encodes just one imp α, whereas humans possess three distinct classes (reviewed in reference 23). The imp α proteins have a characteristic structure consisting of an amino (N)-terminal imp β-binding (IBB) domain of ~100 amino acids (aa) that is followed by 8 to 10 alpha-helical armadillo (ARM) repeats that span most of the remainder of the protein. The ARM repeats are responsible for recognition of the cNLS of cargo (11, 12, 30). The IBB and ARM domains interact to regulate imp α-directed import (30).

Imp β proteins also have a highly repetitive structure. After a short, conserved N-terminal domain, most of the coding sequence consists of up to 20 tandem repeats of the HEAT motif (3). The HEAT and ARM repeats of imp β and α are structurally related motifs (4, 37). HEAT repeats mediate a number of interactions that are critical for the regulation of nuclear import. This domain binds the imp α cNLS adapter, as well as the import regulator Ran, and also mediates interaction with the NPC during transport.

The interactions of imp β with nuclear pore proteins, called nucleoporins (Nups), permit this karyopherin to traverse the NPC. The NPC is a 44- to 60-MDa structure that is composed of ~30 Nups (14, 42). Nearly one-third of the NPC proteins contain variable numbers of hydrophobic phenylalanine-glycine (FG) repeats. Several variants of this repeated motif are found within these FG Nups, including FxFG and GLFG. The FG Nups perform essential functions but individually are somewhat redundant (45). FG Nups are primary components of the central channel and are thought to form the permeability barrier of the NPC by limiting access of the narrow channel in an NPC (17, 18). The ability of karyopherins to bind the FG domains appears to facilitate their transport with their cargo through the NPC (reviewed in reference 46).
Ciliated protozoans such as *Tetrahymena thermophila* have an added challenge for specific nuclear import. These unicellular eukaryotes contain two separate and functionally distinct nuclei in a common cytoplasm (28). The macronucleus contains the somatic genome and is the site of all gene transcription during vegetative growth, while the smaller micronucleus harbors a silent germ line that is passed on to future generations upon conjugation. These two nuclei not only differ in their transcriptional activities and DNA content (the macronucleus is polypliod, the micronucleus diploid) but also replicate their DNA and divide asynchronously. These differences set up the need for regulated distribution of proteins to distinct nuclei. This need is best documented for specific histones. For example, transcription-associated histone variants h1v and h2v, the *Tetrahymena* equivalents of H2AZ and H3.3, respectively, are exclusively found in the somatic macronucleus (1, 2, 48). Furthermore, macro- and micronuclei utilize distinct linker histones (2, 51). Differential nuclear targeting of proteins, beyond the histones, is surely important for the different roles these nuclei play.

To understand how nucleus-specific protein import is achieved in *Tetrahymena*, we have examined the localization of 24 putative karyophorins (13 encoded by imp α-like genes that were initially designated *IMA1* to -13 and 11 encoded by imp β-like genes, *IMB1* to -11). This analysis revealed that while most of the imp α-like family members were specific for one of the two nuclei, the imp β-like proteins primarily localized to both. Nevertheless, a single imp α protein (*Ima10*) was found to be essential; thus, these proteins have nonredundant functions. Furthermore, by localizing putative *Tetrahymena* FG Nups, we found that the NPCs of the micronucleus and macronucleus are assembled with distinct components, which is further evidence that FG repeats define NPC permeability.

**MATERIALS AND METHODS**

**Strains and growth conditions.** *Tetrahymena* cells were cultured at 30°C in SPP or Neff's medium as previously described (5, 24). Cells were starved and matured at 30°C in 10 mM Tris-HCl, pH 7.5. Inbred laboratory strains CU427 [chlt1-1/ chlt-1 (VI, cy-)], CU428 [mprl-1/1-prl-1 (VII, mp-)] and B2066 (II) were used for all transformation and green fluorescent protein (GFP) and yellow fluorescent protein (YFP) fusion localization studies. Micronucleus-defective “star” strains B*(VI)* and B*(VII)* were used to make *IMA10* knockout (KO) heterokaryon strains. Strains are available from The National Tetrahymena Stock Center.

**Identification of putative IMB, IMP, and NUP genes.** *IMA*-encoded candidate proteins were identified by BLASTp searches of the *Tetrahymena Genome Database* (TGD) (http://scq.ciliate.org/cgi-bin/blast-tgd.pl) against The Institute for Genome Resources (TIGR) gene predictions (preliminary gene predictions, August 2004). The protein sequences used in queries were as follows: (i) for imp α, *Saccharomyces cerevisiae* serp1 (GenBank accession gi3102179), Drosophila melanogaster Kap-3 (CGG423-PA) (gi26454525), and Homo sapiens karyopherin α1 (gi87586611); (ii) for imp β, *S. cerevisiae* Kap95 (gi6323579), H. sapiens imp 7 (gi5435998), Mus musculus RanBPS (gi5467916), and Caenorhabditis elegans Imb-2 (gi7555481) and Imb-4 (gi25149087); (iii) for FG Nups, *S. cerevisiae* Nup49 (gi400618), Nup100 (gi400320), Nup116 (gi1346645), and Nup145 (gi1346647). For most gene candidates, the TGD-predicted coding sequence was used to design subsequent functional studies.

**Generation of GFP fusion constructs.** The *IMA10* coding sequence was PCR amplified from *Tetrahymena* genomic DNA, adding XhoI and ApaI restriction sites immediately upstream and downstream of the start and stop codons, and cloned downstream of GFP into vector pIGF-1 (38). To expedite the creation of each GFP fusion construct, a Gateway recombination cassette (Invitrogen) from pEARLYGATE104 (15) was cloned downstream of the GFP coding sequence (named *GFP*-v52). Similarly, the Gateway recombination cassette-YFP fusion from pEarly-Gate101 was cloned downstream of the *MTT1* promoter to generate ribosomal DNA-binding domain-expressed vector pCYT-gtw. For the oligonucleotide primers used to amplify the coding regions of the *IMA*, *IMB*, and *NUP* genes from CU428 genomic DNA, see Table S2 in the supplemental material. The upstream primers began with 5′-CACC after the ATG to guide directional cloning into pENTR/D via directional TOPO cloning (Invitrogen). For some genes, an additional 5′-CTCGAG sequence was added between this 5′-CACC sequence and the start codon, and the sequence 5′-CCCGGG was added after the stop codon during amplification. After verification of the sequence of each clone, the gene was exchanged with the Gateway cassette in pIGF-GTW (IMA and IMB) or pCYC-GTW (NUP) by mixing 100 to 200 ng of a pENTR-IMA/IMBNUP entry clone with ~400 ng of the destination vector and LR Claonase II (Invitrogen) for 16 to 24 h at 25°C. After proteinase K digestion, recombination reactions were transformed into electrocompetent TOP10 Escherichia coli (Invitrogen).

**Generation of IMA chimeric constructs.** An Xho site (underlined) was introduced within the coding regions of *IMA1, IMA10* and *IMA10* by inverse PCR of pENTR-IMA1 -1 and IMA10 with the following primer pairs: *IMA1*, 5′-ATATCTCGAG GATTCCTTATATACCCGCGTAA-3′ and 5′-ATATCTCGAGAAATAGAAAG GTTTCCTTATATACCCGCGTAA-3′; *IMA10*, 5′-ATATCTCGAGCTGTGTTTATACCA-3′ and 5′-ATATCTCGAGCAACTTTCTAAGACC-3′. The first 124 aa of *IMA1* and 132 aa of *IMA10* were then exchanged by digesting the resultant plasmids with XhoI and NotI (a site within pENTR-D) and ligating the appropriate isolated DNA fragments.

After verification of chimeric clones by sequence analysis, the hybrid pENTR clones were recombined with pGFP-gtw as described above. Entry clones containing only the amino-terminal region or the carboxyl-terminal region of either *IMA* or *IMA10* were recombined into pCYT-gtw or pCYT-gtw, respectively.

**Localization of GFP fusion proteins.** GFP and YFP fusions were transformed into *Tetrahymena* sp. strains CU427 and CU428 by conjugative electroporation (19, 20). Transformants were selected by growth in medium containing 100 μg/ml paromomycin sulfate (Sigma, St. Louis, MO). Fusion protein expression was induced for 1 h to 20 h by adding CdCl2 (1 μg/ml) to cultures of log-phase transformants. For conjugative localization, transformed lines were starved in 10 mM Tris-HCl, pH 7.5, containing 0.05 μg/ml CdCl2 for 14 to 20 h. Cells were mixed with equal numbers of starved CU428 or CU427 cells to initiate conjugation, and the concentration of CdCl2 was increased to 0.08 to 0.1 μg/ml. To visualize GFP fluorescence, 0.5 to 1 ml of cells was concentrated by centrifugation at 1,000 × g for 2 to 3 min; 4′-6′-diamidino-2-phenylindole (DAPI) 1 to 5 μg/ml was added to visualize DNA. Cells were mixed with 5 μl of 2% methylcellulose on glass slides and coverslipped. Confocal microscopy was performed on a Nikon E600 upright microscope equipped with a 60× Plan Apo lens (numerical aperture, 1.4), and images were acquired with a Qimaging Retiga EX charge-coupled device camera (Burnaby, British Columbia, Canada) and Openlab software v4.0.2 to v4.0.4 (Improvision).

**Disruption of IMA10 and phenotypic analysis.** The *NEO3* selection cassette (44), which confers resistance to paromomycin, was cloned in place of *IMA10* coding sequence such that targeting this construct via homologous recombination to the *IMA10* locus would remove the first 323 of 520 coding aa of the predicted protein. The cloned upstream *IMA10* sequence spanned nucleotides 475449 to 476464 of scaffold CH445689 (gi62422257), and the downstream region spanned nucleotides 477754 to 478731 (see Table S1 in the supplemental material). ApaI and XhoI restriction sites were added to the upstream fragment, and BamHI and NotI sites were added to the downstream fragment to assist cloning into sites flanking *NEO3* in pMNB1 with the following primers: upstream, 5′-ATAGGCGCTTCTTCTTATCTAATA-3′ and 5′-ATAGCCGCTTCTTATACCCGCTAAT3′; downstream, 5′-ATAGATGCTTATAATATTTATGTG3′ and 5′-ATAGGCTTCTTATACCCGCTAAT3′. The resulting *IMA10* KO cassette, linearized with ApaI and NotI, was introduced into mating CU428 and B2066 cells between 2 and 3 h postmixing with a PDS-1000/He particle bombardment system (Bio-Rad) as previously described (40). Treatment of cells postbombardment focused on nucleolar germ line disruption, and genomic exclusion crosses with *B*(VI) and *B*(VII) strains to generate homozygous heterokaryon lines were all performed as previously described (38). The ratio of the KO to wild-type alleles remaining in polyploid macronuclei was assessed by PCR of crude cell extracts (10) with the
following primers: neo3-3351, 5'-TCGCCCTCTTGACGAGTTCT-3'; IMP_DN, 5'-ATATGAAACTCCATATTGGCAAGCA-3'; IMP_WT, 5'-ATAAGCAGA
AAAGTAAGTAATGCAAAAT-3'.

To generate strains lacking all wild-type IMA10 genes, two mating-compatible IMA10 KO heterokaryons were crossed and individual mating pairs were isolated in growth medium to assess viability, counting live and dead cells 48 h after the start of mating. Cells derived from representative cultures were fixed in 70% ethanol and stained with DAPI to visualize nuclear morphologies.

Reverse transcription-PCR analysis. Total RNA was isolated from growing, starved, and conjugating CU428 and B2086 cells, and reverse transcription-PCR was performed as described previously (38). Amplification was performed on cDNA from 200 ng of total RNA for 28 to 32 PCR cycles at 72°C for 50 s after 30-s denaturation and annealing steps at 94°C and 55°C, respectively. Oligonucleotide primers amplified products from IMA and IMB coding regions that ranged in size from 200 to 400 bp (see Table S3 in the supplemental material). Amplification of α-tubulin (ATU1) confirmed cDNA conversion.

RESULTS

FG repeat-containing Nups differentiate NPCs of micro- and macronuclei. Tetrahymena cells direct many proteins to either the micro- or macronucleus, which suggested to us that the NPCs of these nuclei are different. We searched the Tetrahymena genome for homologues of S. cerevisiae Nups (43, 49) and then further examined the FG Nups. We reasoned that this class of Nups, many of which line the central channel and contribute to NPC selectivity, may differentiate the NPCs of these nuclei. Tetrahymena encodes at least five FG repeat proteins, which we named Nup1 to -5 (see Table S1 in the supplemental material), that vary in predicted size from 405 to 2,017 aa. As C-terminal fusions to yeast Nups have been shown to functionally assemble into the NPC (42), we generated C-terminal fusions of three of these FG Nups (NUP1, -3, and -4) to YFP and examined their localization (Fig. 1). The fusion constructs were maintained in Tetrahymena cells on a ribosomal DNA minichromosome ectopically expressed from the cadmium-inducible MTT1 promoter.

Nup1 is encoded by the smallest open reading frame of the putative FG Nups identified and contains two strings of three and four mixed GLFG and SLFG repeats. Nup1-YFP localized brightly to both micro- and macronuclei (Fig. 1). The fusion protein accumulated to very high levels in the nucleoplasm of the macronucleus, which hampered our ability to visualize any association with the macronuclear membrane and limited the evidence that Nup1 encodes a bona-fide Nup. FG Nups have been shown to localize to both the outer and inner faces of NPCs, in addition to lining the central channel (42). Thus, if we were overexpressing Nup1-YFP, excess protein may have accumulated in the nucleoplasm. Compellingly, Nup1-YFP localized to the micronuclear periphery, consistent with assembly into NPCs.

Nup3 contains a string of 10 repeating GLFG motifs, followed by single FG at intervals of roughly 15 aa. Asparagine-, glycine-, and glutamine-rich regions are denoted by boxes with the percentages of those amino acids indicated.
localized strongly to the periphery of the macronucleus, with relatively minimal accumulation in the nucleoplasm (Fig. 1). Although we expressed Nup3 and Nup1 fusion proteins from the same vector system, the amount of total protein accumulation was specific for each fusion. We detected no association with micronuclei. Localization appears somewhat “patchy” on the macronuclear membrane, which could be indicative of regions with higher or lower densities of NPCs in the focal plane of the image.

Nup4 consists of a 349-aa N-terminal region that is composed of 43.8% asparagine residues and a conserved Nup2 domain (PFam) at its C terminus. While this protein is clearly homologous to Nup98/Nup100, it only contains three FG repeat sequences. In striking contrast to Nup3-YFP, Nup4-YFP localized to the periphery of the micronucleus and was excluded from the macronucleus (Fig. 1). Taken together, these Nup localization studies revealed that the NPCs of these functionally distinct nuclei have unique components. Furthermore, differential localization of the FG Nups implies that specific nuclear transport receptors may exist to target proteins to individual nuclei.

**IMA10 encodes a micronucleus-specific imp α-like protein.**

We suspected that particular *Tetrahymena* karyopherins may have specificity for individual nuclei, as we previously identified a GFP-cDNA fusion protein that localized only to micronuclei and encoded an imp α-like protein named Ima10 (S2). To verify its nuclear specificity, we GFP tagged the entire *IMA10* coding region and examined its localization (Fig. 2). Rapidly upon induction, the GFP-Ima10 fusion protein accumulated to high levels in the cytoplasm. Ima10p was excluded from the macronucleus but localized to the periphery of the micronucleus (Fig. 2A). This is in striking contrast to GFP expressed alone, which accumulates throughout the cytoplasm and the macronucleus but is excluded from the micronucleus (Fig. 2A). This suggests that micronuclear import is very selective while macronuclei are somewhat permeable to macromolecules at least as large as GFP, ~28 kDa.

Ima10 localization changed during the cell cycle. Before cells entered mitosis, this protein was primarily cytoplasmic (data not shown), but as micronuclei began to divide, it localized within micronuclei. *Tetrahymena* underwent cytoplasmic mitosis, and the Ima10 protein accumulated near the nuclear membrane and in the nucleoplasm (Fig. 2B and C), which is consistent with a putative role in transporting cargo into micronuclei. Even as GFP-Ima10 accumulated in dividing micronuclei, cytoplasmic GFP-Ima10 remained abundant in cells. This likely is due to initial overexpression from the strong *MTT1* promoter, as some cells with high levels of GFP-Ima10 showed defects in micronuclear division (see Fig. S1 in the supplemental material). This phenotype does not appear to be caused by the GFP tag, as the fusion protein can rescue a KO of *IMA10* (described below). This overexpression phenotype provided the first clue that Ima10 is critical for micronuclear function.

**IMA10 localizes preferentially to dividing micronuclei.**

The accumulation of GFP-Ima10 in mitotic micronuclei suggested that this protein contributes to the temporal regulation of micronuclear division. If this is so, then Ima10 should be enriched in meiotic, as well as mitotic, nuclei. *Tetrahymena* synchronously undergoes meiosis upon mixing prestarved populations of complementary mating types, initiating conjugation. Typically, mating cells will enter meiosis within 2 h of pairing. *IMA10* transcription is upregulated during this meiosis (see Fig. S2 in the supplemental material), indicating that this protein acts during these events. To explore the connection between *IMA10* expression and meiotic divisions, we examined the localization of the Ima10 protein during conjugation (Fig. 3).

Enrichment of GFP-Ima10 to micronuclei was apparent at the beginning of conjugation (Fig. 3i; see Fig. S2 in the supplemental material for a developmental time line). The protein was highly enriched on and within micronuclei as they completed the first meiotic division (Fig. 3ii to iv). In addition to the meiotic divisions that occur during conjugation, the selected postmeiotic micronucleus in each conjugating cell undergoes one prezygotic mitosis and, after karyogamy to form the zygotic nucleus, two additional nuclear divisions. GFP-Ima10 remained localized to micronuclei through the completion of all postmeiotic mitoses (Fig. 3v and vi). After meiosis and the prezygotic division of selected micronuclei, it appeared to localize preferentially to the gametic nuclei that are exchanged between partners (Fig. 3v and vii). After gametic nuclei fuse in each partner, Ima10 was evenly distributed to the micronuclei through the first postzygotic division (Fig. 3vii) until after the second postzygotic division, when Ima10 was observed to be either enriched on the more posteriorly posi-
tioned nuclei that will remain micronuclei (Fig. 3viii) or was present at equally low levels on both the precursors of the new micro- and macronuclei (Fig. 3ix).

After this last division preceding nuclear differentiation, Ima10 showed mostly cytoplasmic localization, with little association with any nuclei. In some cells, we observed localization to extranuclear membrane fragments that appear to be the by-products of the closed mitoses (white arrows in Fig. 3ix and x). This raises the possibility that the nuclear membrane is remodeled during this last nuclear division in such a way as to lose micronucleus-type NPCs on the precursors of the new macronuclei that allows for incorporation of macronucleus-specific NPCs. We observed similar membrane fragments after other divisions as well (e.g., Fig. 3iv). The rapid switch between NPCs with different specificities during nuclear differentiation warrants further investigation. Nevertheless, the strong localization of Ima10 primarily to meiotic and mitotic nuclei during these developmental stages indicates that this protein likely participates in the import of cargo important for micronuclear division. Alternatively, Ima10p may regulate the assembly of the mitotic spindle, as has been observed for karyopherins in other systems (22, 25, 34, 36).

IMA10 is essential for micronuclear division. In addition to its localization to dividing micronuclei, we observed that cells overexpressing GFP-Ima10 exhibited a delay or failure of micronuclear mitosis (see Fig. S1 in the supplemental material). In the most severe examples, we observed cells that had completed macronuclear division and were undergoing cytokinesis but had undivided micronuclei (in wild-type cells, micronuclear mitosis precedes macronuclear division). This seemed to indicate that precise regulation of micronuclear protein import or other Ima10p function is critical for coordinating division. To examine the possibility that this imp α-like protein is actually required for nuclear division, we disrupted the IMA10 gene by utilizing homologous recombination to replace its coding region with the NEO3 selection cassette (44) (Fig. 4A). Initially, we disrupted the IMA10 gene copies within the somatic macronucleus but were unable to drive the complete replacement of all ~45 wild-type macronuclear copies with the KO cassette by growth under increasingly stringent drug selection, which indicates that Ima10 is essential for vegetative growth. To further study the IMA10 KO (ΔIMA10) phenotype, we disrupted IMA10 in the germ line. This was achieved by introducing the IMA10 KO construct into cells during meiosis. The resulting germ line transformants had one of their two micronuclear IMA10 copies disrupted (i.e., IMA10/ΔIMA10 germ line heterozygotes) and a mixture of wild-type and mutant alleles in the newly formed macronuclei. We then crossed

FIG. 3. Ima10 localizes to diving micronuclei during conjugation. Conjugating cells expressing GFP-Ima10 were examined throughout development, and representative images at specific stages shown are as follows: i, cell pairing (premeiosis); ii to iv, meiosis I; v and vi, postmeiosis, nuclear exchange; vii, first postzygotic division; viii to x, second postzygotic division; xi, macronuclear differentiation. (For a schematic time line of conjugation events, see Fig. S2 in the supplemental material.) White arrows indicate micronuclear membrane fragments that retain GFP-Ima10 after nuclear division. DIC, differential interference contrast.
these strains to generate complete IMA10 KO lines as their progeny had all gene copies derived from their micronuclei (Fig. 4).

To investigate the ΔIMA10 phenotype, we first crossed two lines, each heterozygous in their micronuclei for the disrupted allele (ΔIMA10), and distributed 44 mating pairs into individual drops of culture medium. Meiosis and the subsequent exchange of haploid gametic nuclei result in zygotic nuclei that have the same genetic content in both cells of a mating pair. Thus, all progeny of a single pair will emerge with identical genotypes as cells deplete the parental load of Ima10. All mating pairs from these crosses produced inviable offspring. We could rescue the progeny of these ΔIMA10 heterokaryon crosses by introducing our IMA10-GFP fusion construct during conjugation, which showed that the lethality is due to loss of IMA10 and that our GFP fusion protein used for localization studies is functional.

One day after cells completed conjugation, most of the cells appeared healthy, but by the second day, most of the cells had stopped dividing and had become rounded and heterogeneous in size. On average, each mating pair produced 45 ± 8 cells. In other words, each ΔIMA10 progeny cell could undergo three to four cell divisions after the first caryonidal division that occurs upon exit of conjugation. We fixed cells 24 to 48 h after the completion of conjugation and stained DNA with DAPI to examine the state of the nuclei in the dying progeny. We observed a number of nuclear division defects, including lagging micronuclear chromosomes (Fig. 4C, left), asymmetric division of macronuclei or micronuclei (Fig. 4C, center, right), and abnormal nuclear numbers (Fig. 4C, right). Thus, in addition to aberrant micronuclear divisions, as one would predict for an essential micronucleus-specific imp α protein, we observed macronuclear division defects as well. These macronuclear abnormalities may be caused by secondary alterations of the cell cycle associated with defective micronuclear division.

ΔIMA10 progeny cells fixed and stained with DAPI to visualize nuclear morphologies. White arrows denote lagging chromosomes or abnormal micronuclei. AA, amino acids.

**FIG. 4.** IMA10 is required for micronuclear division. (A) The genomic region of IMA10 is displayed with a double line indicating the coding region of IMA10. Stop or start codons of predicted neighboring genes are indicated. Below is depicted the IMA10 KO construct in which the first 323 (of 520) aa, including the 5′ untranslated region and two of the three ARM repeats (round-edged rectangles), are replaced with the NEO3 cassette. IMA10 was disrupted with this construct via homologous recombination to generate ΔIMA10 cell lines. (B) Strains wild type in their macronuclei but containing ΔIMA10 alleles in their micronuclei were crossed, and the fate of the resulting ΔIMA10 progeny was monitored. Each mating pair produced 45 ± 8 progeny cells, which corresponds to three to four postconjugative divisions before cell death. (C) ΔIMA10 progeny cells fixed and stained with DAPI to visualize nuclear morphologies. White arrows denote lagging chromosomes or abnormal micronuclei. AA, amino acids.

Tetrahymena imp α-like proteins are targeted to specific nuclei. GFP-Ima10 localization to micronuclei suggested that the Tetrahymena genome should encode a macronucleus-specific imp α-like protein as well. To examine this possibility, we searched the TGD sequence for potential imp α homologues. We initially identified 13 TGD-predicted open reading frames, including IMA10, that in reciprocal BLASTp comparisons matched known imp α proteins in the nonredundant database (Fig. 5A), and named these candidate genes IMA1 to -13 (see Table S1 in the supplemental material). IMA1 and -6 showed the greatest similarity to known imp α coding sequences, whereas IMA10 and most of the others were significantly divergent. For all imp α candidates, the primary homology was found in ARM-like repeats (reviewed in reference 23). The relatively large number of imp α-like proteins is quite unusual for a unicellular organism.

We used various sequence analysis tools to examine the domain structures of these candidate proteins (Fig. 5A). Ima1 had a domain structure similar to that of the yeast Srp1 protein, with eight clearly conserved ARM repeats and predicted alpha-helical structure throughout its entire coding sequence. ARM repeats are relatively low-complexity alpha-helical domains; thus, many sequences may be able to form an ARM-like structure without significant sequence identity. Multiple ARM repeats were detectable throughout the other 12 imp α-like coding regions (only clearly conserved ARM repeats are indicated in Fig. 5A), but overall, these protein sequences were much less conserved than that of Ima1. Nevertheless, the initial BLAST searches and comparison of these to the Protein Structure Database with the SWISS-MODEL and PROMAL3D tools revealed that all had significant similarity to imp α proteins (Fig. 5A) and are predicted to have alpha-helical structure throughout their coding regions. Furthermore, the typical
Nucleoplasmin showed strong similarity to impIMA9ies (Fig. 6C) (29). Furthermore, even though Ima16 (44a) and its localization to basal bodies (Fig. 6) was subsequently renamed SPG6, are on the left. The scale indicates the predicted protein length (amino acids). IBBput, putative IBB. The regions of each that show recognizable structural similarity to impα in the Protein Structure Database are shaded. The alignment of the putative IBB domains in each impα-like gene is shown in panel B. The consensus positions listed are found in at least half of the aligned sequences. The position of the starting spacing between recognizably conserved ARM repeats was observed to be in increments of one or multiple repeat lengths (≈50 aa), which may indicate structural homology even in the absence of primary sequence similarity.

Of these 13, only Ima5 had a recognizable IBB domain in our initial bioinformatic analyses. However, realignment of the N-terminal regions of multiple candidates with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/) identified a conserved basic sequence in eight (Ima2 to -5, -8, and -10 to -12) of the 13 candidates that is similar in composition to the IBB domain of impα proteins (Fig. 5B). Ima1 was also found to contain a similar basic domain in its N terminus, but it did not conform as well to this putative IBB consensus found in the more divergent impα-like proteins. The difference between the putative IBB domains in Ima1 and these other candidates appears to be, in part, responsible for their distinct targeting to macronuclear and micronuclear, respectively (Fig. 6). The identification of the IBB-like domain together with the ARM repeats supports the tentative assignment of these nine as impα-like proteins.

Not all of these 13 likely function as impα proteins. For example, IMA7 was subsequently renamed SPG6 because of its similarity to H. sapiens SPAG6 (gi49065530) and Chlamydomonas reinhardtii PF16 (44a) and its localization to basal bodies (Fig. 6C) (29). Furthermore, even though SPG6, IMA6, and IMA9 showed strong similarity to impα proteins within their ARM repeat regions, these candidates did not have an apparent IBB domain, and the arrangement of the first ARM domain within <50 aa of their amino termini is not consistent with impα structure.

If these predicted proteins function in nuclear import, they should localize to nuclei. We GFP tagged each as we had done for IMA10 and examined their localization. Most of the GFP fusion proteins accumulated to high levels in the cytoplasm. More importantly, and consistent with their predicted nuclear transport activity, we observed that the majority also localized to one or both types of nuclei (Fig. 6B). The most conserved impα-like protein, Ima1, localized strongly to macronuclei (Fig. 6A). This protein is expressed constitutively through growth and development (see Fig. S2 in the supplemental material). Together with its domain structure and localization, Ima1 appears to be the primary impα-like protein directing macronuclear import. Ima6 was also exclusively localized to macronuclei in growing cells, but it lacked a putative IBB domain and contained a nucleoplasmin domain within its C-terminal half and thus is not a clear impα homologue. This does not exclude a possible role for this ARM repeat protein in macronuclear import.

As the macronucleus provides all of the cell’s gene expression, we were somewhat surprised to find that, including Ima10, 9 of the 13 ARM repeat proteins (Ima2, -3, -4, -5, -8, -10, -11, -12, and -13) localized to micronuclei (Fig. 6B). These GFP-Ima fusions were primarily restricted to the nuclear periphery, appearing as a ring surrounding the dense, DAPI-staining interior (Fig. 6B). This is in contrast to the GFP-micronuclear linker histone, which localized strongly within the
chromatin-rich nucleoplasm (52). Most of the micronucleus-localized Ima proteins showed very strong cytoplasmic accumulation (Fig. 6B, panels A2, A3, A4, A5, A8, A10, and A13), which initially made the localization pattern around the micronuclear periphery challenging to interpret. The micronucleus sits in an invagination of the macronuclear membrane prior to mitosis; thus, the ring of fluorescence around the micronucleus could result from cytoplasmic GFP filling the gap between the two nuclei when viewed in the focal plane of the micronucleus. By examining cells during mitosis or during conjugation, when the nuclei are apart, we were able to confirm the micronuclear localization of all of the candidates except Spg6 and Ima9 (data not shown). For these two, we detected localization to basal bodies and to the oral apparatus (white arrows in Fig. 6C). Given that these also lack a putative IBB domain, they likely do not participate in nuclear import.

The localization patterns (e.g., the ratio of cytoplasmic to nuclear localization) of the individual micronucleus Ima fusion proteins varied. Ima8, -10, -12, and -13 typically exhibited nuclear fluorescence above cytoplasmic levels, while Ima2, -4, and -5 had signals equal to cytoplasmic levels. As all of these proteins are ectopically expressed from the MTT1 promoter,
specific patterns may reflect different import-export rates or overall cargo abundance. Two of the micronucleus-localized imp α-like proteins, Ima3 and -12, also exhibited localization to macronuclei within the first few hours after the induction of expression. However, the stable protein remaining after overnight culturing was predominantly lost from macronuclei (Fig. 6B; data not shown). It is possible that the macronuclear localization observed was an artifact of initial overexpression and, at equilibrium, these importins are targeted to micronuclei. With the possible exception of these two candidates, the GFP-Ima fusion proteins associated preferentially with either the macronucleus or the micronucleus. These data lead us to suggest that the imp α-like proteins, together with specific FG Nups, significantly contribute to the specificity of the Tetrahymena nuclear import system.

**Imp α proteins are directed to specific nuclei by both the putative IBB and cargo binding domains.** While the highly structured ARM repeats of imp α proteins bind cNLS containing cargo, the IBB domain regulates cargo binding and mediates the formation of the cargo/imp α/imp β ternary complex (see reference 23). To test the role of putative IBB domains (Fig. 5) in nuclear targeting, we exchanged the N termini of the macronucleus-specific Ima1 and micronucleus-specific Ima10 proteins. These Ima1/10 chimeras exchanged sequences downstream of codon 124 (IMA1) or 132 (IMA10), which is the beginning of the first ARM repeat of each. IMA1 with the IMA10 N terminus (IMA1N10) and IMA10 with the IMA1 N terminus (IMA10N1) were fused to the GFP coding sequence, and their localizations are visualized (Fig. 7).

Whereas the original Ima1 and -10 GFP fusions showed very specific macronucleus or micronucleus targeting (Fig. 6A and B), respectively, both Ima1N10 and Ima10N1 exhibited dual localization (Fig. 7B). Thus, the putative IBB domains in the N termini of Ima1 and Ima10 was sufficient to provide novel nuclear targeting of the predicted NLS binding domain of each to different nuclei. Nevertheless, the N-terminal region was unable to direct exclusive targeting of the chimeras. Ima1N10 was still efficiently targeted to its original macronuclear destination, but it also acquired new specificity for the micronucleus. Similarly, GFP-Ima10N1 was localized to macronuclei, as well as its original target, the micronucleus. While it is difficult to quantify, it generally appeared that the original target nucleus of the cargo domain was the preferred target for the fusion protein (compare relative abundance in the two nuclei for each chimera in Fig. 7B).

While still able to localize to micronuclei, cells expressing the GFP-Ima10N1 chimera exhibited micronuclear division defects very similar to those observed upon the overexpression of GFP-Ima10 (Fig. 7B; see Fig. S1 in the supplemental material). In addition, the chimeric protein accumulated in one or more discrete foci at the periphery of micronuclei. These micronuclei were often misshapen, having very angular nuclear membranes (Fig. 7B). This appears to indicate that this protein interferes with the normal function of the nuclear division machinery.

We also generated separate fusions of the IMA1 and IMA10 cargo binding domains to the C terminus of GFP and their IBB domains to the N terminus of YFP. Both domains of IMA1 were alone sufficient to target the fluorescent protein exclusively to macronuclei (Fig. 7C). The IBB domain of IMA10 fused to YFP was not expressed to detectable levels, but the ARM domain targeted GFP primarily to micronuclei, with some accumulation in macronuclei as well (Fig. 7C). The behavior of the chimeric Ima proteins, along with that of these individual domain fusions, implies that exclusive targeting of nuclear proteins requires the interaction of the ARM repeats with their NLSs and interaction of the imp α IBB domain with an appropriate imp β protein.

**Tetrahymena imp β proteins localize to both micro- and macronuclei.** The putative IBB domains in these imp α-like proteins suggest that they carry their cargo through the NPCs by associating with particular imp β proteins. To identify the full repertoire of karyopherins in Tetrahymena, we queried the genome sequence with six known imp β protein sequences and identified 11 putative imp β (IMB)-related genes (Fig. 8A). Our search for conserved domains in these candidates revealed that IMB candidates 9 and 10 contained Xpo protein domains, and reciprocal comparisons against GenBank sequences identified known exportin proteins as the best matches. Hence, we renamed their genes XPO1 and XPO2 (Fig. 8A). All 11 IMB genes are expressed, and each showed uniform mRNA levels during growth and throughout conjugation (see Fig. S2 in the supplemental material). The steady-state mRNA level of each IMB gene was found to decrease when cells were cultured under starvation conditions, but this is expected for most Tetrahymena genes. Overall, IMB gene expression showed less variability than observed for the IMA genes.

We somewhat expected nucleus-specific Imb localization that would match the imp α-like proteins. Counter to this expectation, the majority of the GFP-Imb fusion proteins localized to both nuclei, with the exceptions being Imb3 and -4, which localized exclusively to macronuclei (Fig. 8B). The two exportin-related proteins also localized to both nuclei. Most of the macronucleus- or dual-localized GFP-Imb fusions accumulated to the greatest extent at the macronuclear envelope (the high degree of cytoplasmic accumulation of GFP-Imb2 and -11 obscured any enrichment). This membrane association is consistent with these proteins acting in nuclear import.

Even among the six imp β-like proteins that localized to both micronuclei and macronuclei, the relative abundance of cytoplasmic accumulation and distributions between nuclei varied. Imb1, -5, -6, and -8 exhibited higher accumulation on or in macronuclei than within the cytoplasm, whereas Imb7 and -11 accumulated equally strongly in the cytoplasm and the two nuclei. Imb2 had very strong cytoplasmic accumulation and relatively weak localization to either nucleus. Imb6 was unique in that it showed stronger localization to micronuclei than to macronuclei. The diversity of localization patterns of these imp β proteins, all expressed from the same vector system, seems to indicate that they likely transport different cargos that can vary widely in their abundance and transport rates.

The two exportin homologs encoded by XPO1 and XPO2 also exhibit distinct localization patterns. GFP-Xpo1 accumulated on or within the macronucleus and the micronuclear envelope; GFP-Xpo2 was predominantly cytoplasmic. These two proteins clearly have different subcellular distributions during growth, which likely reflects distinct equilibria in their transport functions. When taken together, the diverse localization patterns of both the imp α- and imp β-like proteins indicate extensive functional specialization within these large...
karyopherin families, which is likely required to coordinate the respective activities of these distinct nuclei.

**DISCUSSION**

*Tetrahymena* encodes a large number of karyopherins. The diversification of the imp α family is particularly striking. In this study, we characterized 13 ARM repeat-containing proteins, 11 of which localized to micro- or macronuclei and likely participate in nuclear import and/or regulate other nuclear processes. Additional bioinformatics indicates that *Tetrahymena* encodes at least four additional candidates, two of which possess the conserved IBB-like domain found in eight of nine micronucleus-specific ImA proteins (Fig. 5B). The diversification of the imp α-like karyopherins in this ciliate is in dramatic contrast to the situation in *S. cerevisiae*, which encodes a single imp α protein. Even the metazoan *C. elegans* encodes only three imp α homologs (21). The

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**FIG. 7.** Chimeric ImA1 and -10 proteins localize to both micro- and macronuclei. (A) Diagrams of IMAI (the wide, single line), IMAI0 (the double line), and chimeras IMAI1N10 and IMAI0N1 display the relevant amino acid (AA) positions and conserved Arm repeats (round-edged rectangles). (B) Representative localizations of the GFP-chimeric proteins are displayed as a series of differential interference contrast (DIC), DAPI-stained, and GFP fluorescent images as indicated. Micronuclei are denoted by white arrowheads. (C) Localization of the IMAI IBB domain fused to the N terminus of YFP (IMAI1N) and the ARM domains of IMAI and -10 fused to the C termini of GFP, IMAI1C, and IMAI0C, respectively. DAPI-stained DNA, blue; fluorescent protein localization, green.
extensive divergence in coding sequence, the variable expression patterns (see Fig. S2 in the supplemental material), and the finding that IMA10 is essential suggest that the different Tetrahymena imp/\textit{H9251}-like proteins are not simply redundant in function. It is compelling to speculate that the expansion of this gene family is directly related to the need to accurately import proteins to the two functionally distinct nuclei for the regulation of cellular processes. As macronucleus-targeted Im1 is much more similar in sequence to imp \(\alpha\) proteins of other eukaryotes than to the divergent micronucleus-localized Ima proteins, we conclude that the macronucleus import machinery has preserved greater similarity to conventional cNLS-directed import. In support of this,
heterologous nuclear proteins are specifically imported into Tetrahymena macronuclei (50).

The number of imp β-like proteins is similar to that of many eukaryotes. The fact that these proteins localize predominantly to both nuclei (Fig. 8) seems to indicate that these are involved largely in the transport of cargos that are common to both nuclei. It appears likely that either the macronucleus-specific Imb3 or -4 protein acts with Ima1 to import proteins into macronuclei. None of the imp β-like proteins were clearly micronuclear specific; although Imb6 was preferentially associated. It is possible that micronuclear imp α proteins may transport their cargo through the pore without a collaborating imp β protein (33, 39). However, when we switched N-terminal domains between Ima1 and -10, we found that both chimeras localized to micro- and macronuclei. This finding suggests that these putative IBBs interact with imp β proteins and leads us to believe that micro- or macronuclear specificity is conferred by the ternary complex of cargo/imp α/imp β.

One implication of such a large number of micronucleus-specific imp α proteins is that micronuclear functions are regulated, in part, by protein import. We show that loss of IMA10 leads to micronuclear division defects (Fig. 3). Overexpression of GFP-Ima10p also delayed or blocked division (Fig. 1; see Fig. S1 in the supplemental material), possibly due to the sequestration of bound cargo in the cytoplasm, which further demonstrates that this imp α protein regulates micronuclear mitosis and meiosis. Studies with several systems have linked imp α proteins to the control of nuclear division (22, 25, 34, 36). During mitosis and particularly meiosis, we observed that GFP-Ima10 localized to foci within micronuclei. These Ima10 foci could represent association with the spindle or kinetochore, which has been observed for several nuclear transport proteins in diverse eukaryotes (reviewed in reference 27). The micronuclear and macronuclear cell cycles in Tetrahymena are asynchronous. Regulated protein import may be one mechanism this ciliate uses to coordinate these events. The finding that an imp α-like protein is essential for nuclear division in Tetrahymena, as has been shown in other organisms, suggests that a role for these proteins in coordinating mitosis may have arisen very early in the evolution of eukaryotes.

We are intrigued that the NPCs of the individual nuclei have unique components, as demonstrated by the differential localizations of Nup3 and -4 to macro- and micronuclei, respectively (Fig. 1). While these are both FG Nups that are related to Nup98/Nup100, they clearly have sequences divergent from each other. Compared to other eukaryotic FG Nups, micronucleus-associated Nup4 is more divergent in sequence than macronuclear Nup3. Likewise, the micronuclear imp α-like proteins are more divergent relative to Ima1, observations that further argue that import into micronuclei has become highly specialized. We speculate that the various FG Nups have distinct transport affinities for specific karyopherins, making the channels through the NPCs of the micro- and macronuclei differentially permeable to importin-cargo complexes. We do not know the full extent of the unique NPC components of the different nuclei. In our bioinformatic analyses, we did not see any evidence for two complete but divergent sets of Nups encoded in the genome. The NPC differences could be limited to a small number of components that interact with the karyopherins during transport. The observation that FG Nups are differentially associated with discrete nuclei further indicates that FG repeats serve as major mediators of NPC permeability (45, 46).

While some studies have indicated that NPCs of different tissues have unique components (47), Tetrahymena appear to be able to assemble distinct NPCs in a single cell. We do not know how this is achieved, but it is likely a dynamic process. This is most apparent during a short period (<1 h) of conjugation immediately following the second postzygotic nuclear division, when the four micronucleus-derived zygotic nuclei differentiate into two micronuclei and two macronuclei (Fig. 3vii to xi). The new macronuclei must quickly lose their micronuclear NPCs and acquire new macronuclear NPCs. This may occur by discarding membrane during division, as we observed extranuclear membrane remnants that were associated with GFP-Ima10 (Fig. 3). This stage of Tetrahymena development should provide a unique opportunity with which to investigate new NPC assembly in vivo.

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