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Expression and cDNA Cloning of *klp-12* Gene Encoding an Ortholog of the Chicken Chromokinesin, Mediating Chromosome Segregation in *Caenorhabditis elegans*

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In eukaryotes, chromosomes undergo a series of complex and coordinated movements during cell division. The kinesin motor proteins, such as the chicken Chromokinesin, are known to bind DNA and transport chromosomes on spindle microtubles. We previously cloned a family of retrograde C-terminus kinesins in *Caenorhabditis elegans* that mediate chromosomal movement during embryonic development. Here we report the cloning of a *C. elegans klp-12* cDNA, encoding an ortholog of chicken Chromokinesin and mouse KIF4. The KLP-12 protein contains 1609 amino acid and harbors two leucine zipper motifs. The *in situ* RNA hybridization in embryonic stages shows that the *klp-12* gene is expressed during the entire embryonic development. The RNA interference assay reveals that, similar to the role of Chromokinesin, *klp-12* functions in chromosome segregation. These results support the notion that during mitosis both types, the anterograde N-terminus kinesins such as KLP-12 and the retrograde C- terminus kinesins, such as KLP-3, KLP-15, KLP-16, and KLP-17, may coordinate chromosome assembly at the metaphase plate and chromosomal segregation towards the spindle poles in *C. elegans*.

Keywords: *C. elegans*, Cell division, Chromokinesin, Chromosomal segregation, KLP-12, Leucine zipper motif.

Introduction

Cell division is basic to life propagation. During cell division, chromosomal movement is a highly complex and dynamic process, including chromosome assembly at the metaphase plate and their segregation towards the opposite spindle poles. However, little is known about the molecular nature of forces that are required for the chromosomal locomotion.

The hallmark of kinesin motor proteins is the conserved globular motor domain, consisting of about 350 amino acids that contain ATP and microtubule binding sites. The globular motor domains are linked to the tail domains (via a stalk domain) of various sizes and shapes and are believed to specify cargo for different motors. In conventional kinesin heavy chain (KHC), as well as in most kinesin like proteins (KLPs), the motor domains are located in the amino terminus of the proteins. Most of the N-terminus motor kinesins are anterograde motors; *i.e.* they move cellular cargo from the minus end of the microtubule to the plus end. Several other KLPs, however, were discovered in which the conserved motor domain is located in the carboxyl terminal of the protein. The C-terminus kinesins are known to move in a retrograde manner. For example, they carry the cellular cargo from the plus end of the microtubule to the minus end. In short, kinesin motor proteins can participate in moving cellular cargo both in the anterograde and retrograde directions on microtubule tracks (see reviews, e.g., Bloom and Endow, 1994; Wright and Scholey, 1992; Hirokawa, 1998; Endow, 1999).

Different subfamilies have been recognized within the kinesin super-family, with members of each subgroup showing a high homology in the motor domain, but relatively less sequence homology in the non-motor domain. Members of each subfamily, however, appear to perform a similar cellular function. A combination of molecular and genetic approaches revealed the existence of several kinesin motor proteins that participate in the normal spindle orientation and distribution of chromosomes.

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The kinesin proteins, which have been proposed to affect spindle function and chromosome locomotion, include the *yeast* BimC subfamily of KLPs. These are N-terminus anterograde motors and could move toward plus ends of spindles in an opposite orientation, thus pushing them into separate poles. Mutants in *bimC* in *Aspergillus nidulans* fail to divide and instead display monopolar spindles and highly polyploid cells as a result of the extra duplication of chromosomes. This is followed by the inability of normal spindles to align and distribute chromosomes (Enos and Morris, 1990). Subsequently, similar genes were identified in various organisms, such as in *Schizosaccharmyces pombe (cut7)* (Hagen and Yanagida, 1990), *Saccharomyces cerevisiae (CIN8 and KIP1)* (Hoyt *et al.*, 1992; Roof *et al.*, 1992), *Xenopus laevis (Eg5)* (Sawin *et al.*, 1992), *Drosophila melanogaster* (KLP61F) (Heck *et al.*, 1993). An important point here is that while CIN8 and KIP1 are redundant motors in *S. cerevisiae*, the KLP61F is essential for development in *D. melanogaster* (Heck *et al.*, 1993). The KLP61F kinesin cross-links microtubules within interpolar microtubule bundles of *Drosophila* embryonic mitotic spindles (Sharp *et al.*, 1999).

The MKLP1 (Nislow *et al.*, 1992) and CHO1 (Sellitto *et al.*, 1988) are anterograde KLPs that are required in the sliding of the spindle midbody during the later stages of anaphase, which is necessary for spindle elongation and chromosome segregation. The *C. elegans* ZEN-4, a member of the MKLP1 family, plays a direct role in cytokinesis and midzone microtubule organization. Mutants in *zen-4* form multinucleate single-celled embryos as they proceed to cycle through mitosis, but fail to complete cell division (Raich *et al.*, 1998). The ZEN-4 protein was shown to associate with the spindle midzone during anaphase and persists during cytokinesis at the midbody region, suggesting its role in linking the midzone microtubules (Raich *et al.*, 1998). Noteworthy among anterograde kinesin motors that are involved in chromosomal movement, are the members of the Chromokinesin subfamily. The chicken Chromokinesin contains both the conserved ATP and microtubule binding motor domain, as well as an atypical basic-leucine zipper DNA binding motif (Wang and Adler, 1995). Similarly, retrograde kinesin motors in various organisms were implicated in chromosomal distribution during mitosis and meiosis. For example, one of the well-characterized kinesin motors is the nonclaret disjunctional (Ncd), a retrograde C-terminus motor protein that is required for normal chromosome distribution in *Drosophila* oocytes and early embryos (Endow *et al.*, 1990). Mutants in *ncd* are affected in gametic and early zygotic chromosome non-disjunction and loss. They also produce high levels of embryonic lethality and aneuploidy, as first observed by Sturtevant (1929). The Ncd protein has been shown to be associated with spindles in dividing cells (Hatsumi and Endow, 1992; Bloom and Endow, 1994). We previously described four C-terminal motor kinesins in *Caenorhabditis elegans* (*klp-3, klp-15, klp-16* and *klp-17*) that were proposed to mediate chromosome movement during the early embryonic development (Khan *et al.*, 1997; Ali and Siddiqui, 2000; Ali *et al.*, 2000).

The *C. elegans unc-116* gene encoding the kinesin heavy chain was shown to affect the spindle orientation and cell division at the 1-cell stage embryos (Patel *et al.*, 1993; Thierry-Mieg, D., White, J., Hedgecock, E., Hall, D., Ali, M. Y., Siddiqui, S.S. unpublished observations). The advantage of investigating the kinesin superfamily function in the nematode *C. elegans* stems from the fact that the simple animal is amenable to both classical and molecular genetics. Furthermore, an extensive account of the *C. elegans* development is known and its entire genomic sequence is available (Sulston *et al.*, 1983; The *C. elegans* Sequencing Consortium, 1998).

In this paper we report the molecular cloning of a novel *klp-12* cDNA encoding the largest kinesin-like protein KLP-12. It is comprised of 1609 residues in *C. elegans.* KLP-12, a N-terminus kinesin that shares a significant homology with the chicken Chromokinesin (Wang and Adler *et al.*, 1995) as well as the mouse KIF4 (Sekine *et al.*, 1994; Lyu *et al.*, 1997), also harbors two leucine zipper motifs in the stalk domain. By using RNA *in situ* hybridization and the RNA interference assay, we determined the pattern of *klp-12* expression during embryonic development. Our data suggest that the KLP-12 expression is associated with chromosomes during mitosis and a lack of *klp-12* function results in embryonic lethality. In the arrested embryos, the cell division is blocked and the cellular nuclei show polyploid chromosomes. The cloning of the *klp-12* gene thus provides a novel reagent to elucidate the molecular basis of chromosome segregation during early development.

Materials and Methods

Isolation of the cDNA clones encoding kinesin like proteins A *C. elegans* cDNA library in a λ-ZAPII vector (kindly provided by Y. Kohara, National Instt. of Genetics, Mishima) was screened using a digoxinenin labeled probe that was made from a truncated cDNA clone yk169e9 of size 1860 bp that corresponds to the *klp-12* gene (cosmid T01G1.1). From a screen of about 2×10^6 plaques, two positive cDNA clones for the *klp-12* gene were identified. The insert was excised from the phage as a pBluescript vector using standard protocols (see Khan *et al.*, 1997). The nucleotide sequence of the cDNA clones was determined by the dideoxy chain termination method (Sanger *et al.*, 1977). The clones were PCR amplified using a cycle sequencing kit (Epicentre Technologies co.). The PCR products were analyzed using an automatic DNA sequencer (Pharmacia ALFexpress). Both clones were sequenced. The clone SQ#Y112 is a full-length cDNA clone of a size 5243 bp and the other clone had missing upstream sequences at the 5ĺ end.

Northern Blot Analysis The total RNA was extracted from the wild-type mixed stage (larvae and adult) worms according to the kit vendor's instructions (Qiagen, Germany) with minor

modifications for *C. elegans*. The PolyA$^+$ RNA was prepared from the total RNA using Oligotex-dT30 according to the kit manufacturerís instructions (Takara Biochemicals, CA). A 10-μg amount of polyA$^+$ RNA was electrophoresed on a denaturing agarose gel and transferred to a nylon membrane. For cross-linking, the membrane was irradiated by UV light for five minutes and baked at 80°C for two hours. The filter was then hybridized at 65°C for 16 hours (as described by Khan *et al.*, 1997) with digoxigenin-labeled probe (made using *klp-12* cDNA). The hybridization buffer contained 5×SSC, 2% (w/v) blocking reagent, 0.1%(w/v) N-lauroylsarcosine, and 0.02% (w/v) SDS. The probed filter was washed twice with 2×SSC (0.1% SDS for five minutes) and twice with 0.1×SSC, (0.1% SDS for 10 minutes each). The filter was exposed to X-ray film for two hours. The signal was detected using the kit manufacturerís instruction (Boehringer Mannheim). A 5.4 kb band that corresponded to the *klp-12* was detected. The RNA size marker (Takara Biochemicals) was used to measure the size of the detected signal.

Protein homology search and computer based structure prediction The overall homology was searched using the BLAST program (Altschul, *et al.*, 1990). The CLUSTALW program (Thomson *et al.*, 1994) was used to search protein homologies between KLP-12 and other proteins.

Secondary structure prediction The secondary structure of KLP-12 was deduced using the published algorithm (Frishman *et al.* 1996). The probability of a coiled coil structure of KLP-12 was determined using a published program (Lupas *et al.*, 1991).

RNA *in situ* hybridization For the *klp-12* RNA *in situ* hybridization in embryos, we followed the published method (Tabara *et al.*, 1996). The DIG labeled *klp-12* cDNA (SQ#Y112) was used as a probe for hybridization. The stained samples were observed using a Nikon E800 Microscope with a Nomarski optics. The experiment was repeated five times in order to confirm the reproducibility of the results. As controls, *dpy-20* (Clark *et al.*, 1995) cDNA and bluescript vector DNA probes were used, which showed no staining.

RNA Interference assay The Double stranded (**ds**) RNAi experiment was done according to the published method (Fire *et al.*,1998). In short, Plasmid DNA for SQ#Y112 was isolated and purified according to the kit manufacturer's instruction (Qiagen). Sense and antisense strands of RNA were synthesized using T3 and T7 RNA polymerase (Novagen, Inc.) respectively. An equal amount of the sense and anti sense RNA were mixed in a 3× Injection buffer (20 mM KPO4, pH 7.5, 3 mM K Citrate, pH 7.5, 2% PEG 6000) and incubated at 68°C for 10 minutes and then 30 minutes at 37°C. The concentration of the double stranded RNA was measured by determining the OD260. The RNA was electrophoresed on a standard 1% agarose gel. The dsRNA was injected into the gonad of a young adult and the L4 larvae of a wild type hermaphrodites at a concentration of ~500 ng/μl. Injected animals laid few unaffected progeny which were presumably fertilized before injection. The effect of the RNAi was most significant after16-24 hours of injection. After the injection, the injected animals, as well as the resulting embryos, were fixed and permeabilized with the Carnoys solution (ethanol: acetic acid: chloroform = 60 : 30 : 10) and then incubated with DAPI for 20 minutes. After this the embryos were observed under the Nomaraski (DIC) microscope. In controls, only the injection buffer and *osm-3* (Shakir *et al.*, 1993; Tabish *et al.*,1995) dsRNA was injected into the developing gonads and they produced normal progeny embryos with no morphological or developmental defects.

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence data bases with the accession number **AB035591** (clone SQ#Y112) corresponding to *klp-12* gene.

Results

Isolation of a *klp-12* cDNA clone and the Northern blot analysis The genome consortium has placed the cosmid T01G1 on the chromosome IV that encodes *klp-12* gene (Wilson *et al.*, 1994). The *klp-12* gene maps in the region between *unc-31* and *unc-30* loci on the genetic map of *C. elegans* (Fig. 1 A). In order to obtain a full-length cDNA clone, we screened a *C. elegans* cNDA library in the λ-ZAPII vector using a DIG-labeling probe and identified a single 5243 bp clone (SQ#Y112) with an open reading frame. Sequencing the SQ#Y112 clone reveals that the 5243 bp clone (Fig. 1D) is indeed derived from the *klp-12* gene and encodes the complete transcript. Northern blot analysis was performed from the wild type polyA$^+$ containing RNA, using the SQ#Y112 cDNA clone as a probe that detects a 5.4 kb band corresponding to the *klp-12* gene (Fig.1B). This would suggest that the SQ#Y112 clone is a full-length clone that contains the entire *klp-12* gene. The genomic sequence (T01G1.1) of *klp-12* spans the 19210 bp that includes 23 exons interrupted by 22 introns (Fig. 1C). The start and stop codons are located at 11 bp and 4838 bp of the sequence. The poly-adenylation site is located at 99 bp downstream of the stop codon (Fig. 1D).

KLP-12 is a kinesin like protein, an ortholog of chicken Chromokinesin and mouse KIF4 The deduced amino acid sequence, based on the *klp-12* cDNA sequence data, reveals a protein of 1609 amino acids (Fig. 2A). The KLP-12 protein shares a high homology (49%) in the motor domain region with chicken Chromokinesin (Wang and Adler, 1995) and mouse KIF4 kinesin (Sekine *et al.*, 1994). The overall homology, however, is quite low, only 22% (Fig. 3). The 3D crystal structure of the *Drosophila* kinesin Ncd has already been determined (Sublin *et al.*, 1997). Based on their structural data, we found that only one ATP nucleotide binding pocket (N4), the KLP-12 motor domain, contains all of the nucleotide binding pockets (N1, N2, N3) and the microtubule binding (MT) site (Fig. 2D).

Based on the secondary structure, we found that the KLP-12 has three major domains. There is a globular motor domain (residues 1-376) that is located in the N-terminus. It consists

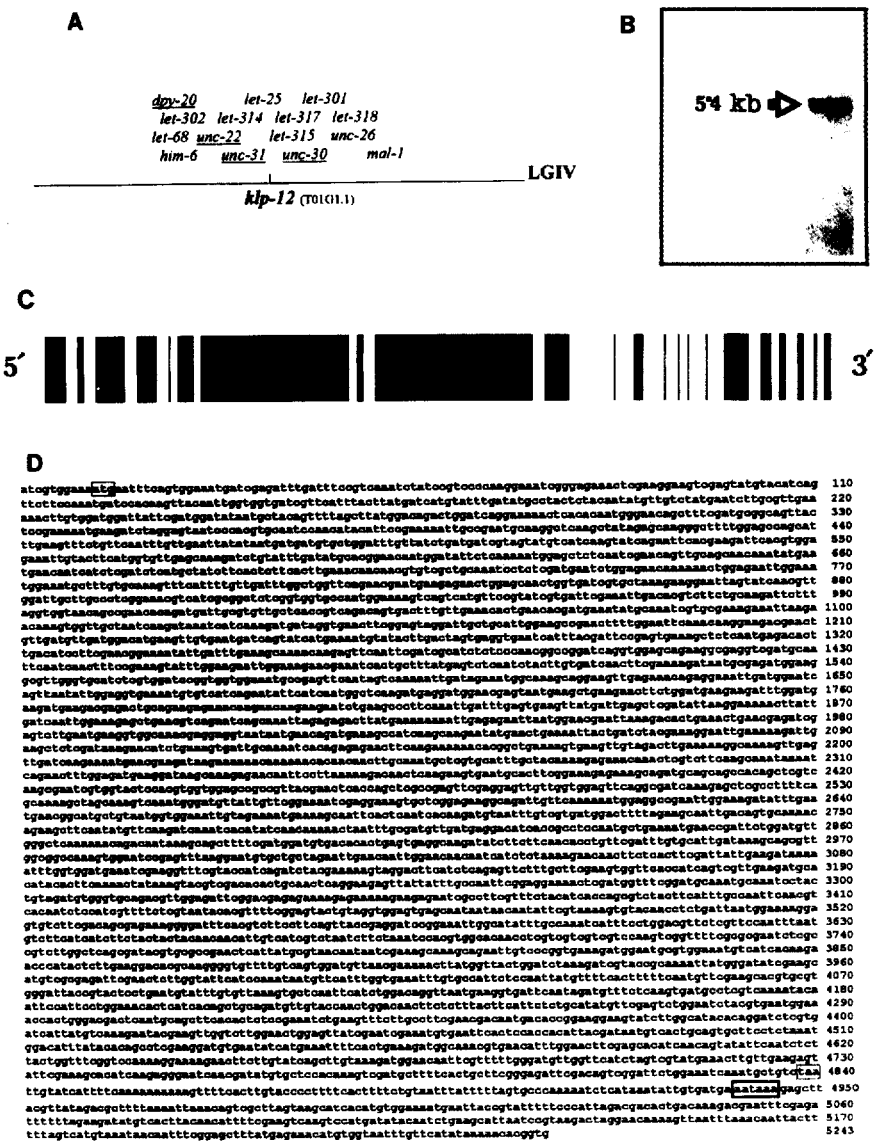

Fig. 1. A. Genetic and physical mapping of the *klp-12* gene on chromosome IV. **B.** Northern blot analysis of the *klp-12* mRNA. A 5.4 kb band is seen in the Northern blot using 10 μg of N2 (wild type) mixed mRNA, probed with the *klp-12* cDNA (SQ#Y112). **C.** *klp-12* gene is encoded by 23 exons and 22 introns, total length of the gene is 19210 bp and the spliced DNA sequence is 4830 bp. **D.** The nucleotide sequence of the full-length cDNA clone. The start codon **atg** and the stop codon **taa** are located at 11 bp and 4838 bp respectively, and are indicated by a single borderline. The consensus polyadenylation site **aataaa** is indicated by a double borderline, located at 99 bp downstream of the stop codon.

of the alternating alpha helix, beta sheet and loop (Fig. 2A-B and E). The domain followed by the motor domain is known as a stalk domain, which is a alpha helical coiled coil structure (residues 377-1045) (Fig. 2A-C and E). The structure of the tail domain (residues 1247-1609) is globular and located in the C-terminal of the protein (Fig. 2A-B and E). The KLP-12 contains a putative coil domain (residues 1046-1246) that connects the stalk domain and the tail domain (Fig. 2A-B). Compared to the mouse KIF4 and chicken Chromokinesin, the KLP-12 has an unusually large tail domain (Fig.2B). In the KLP-12, the highly conserved motor domain signature sequence is present at residues 259-270. The KLP-12 contains two nuclear localization signals at residues 820-826 and 725-741 in the stalk domain (Fig. 2A).

It is interesting that the two leucine zippers DNA binding motif sites are present in KLP-12 at residues 419-440 and 426-447 in the coiled coil stalk region (Fig. 2A). It is proposed that the Chromokinesins directly bind their DNA cargo and may function to mediate chromosome movement to the metaphase plate during cell division. Although the *C. elegans* KLP-12 shares a significant homology in the motor domain with the members of other Chromokinesin, the structure of its tail is not so conserved and there appears to be a significant variation in the tail regions among the members of the

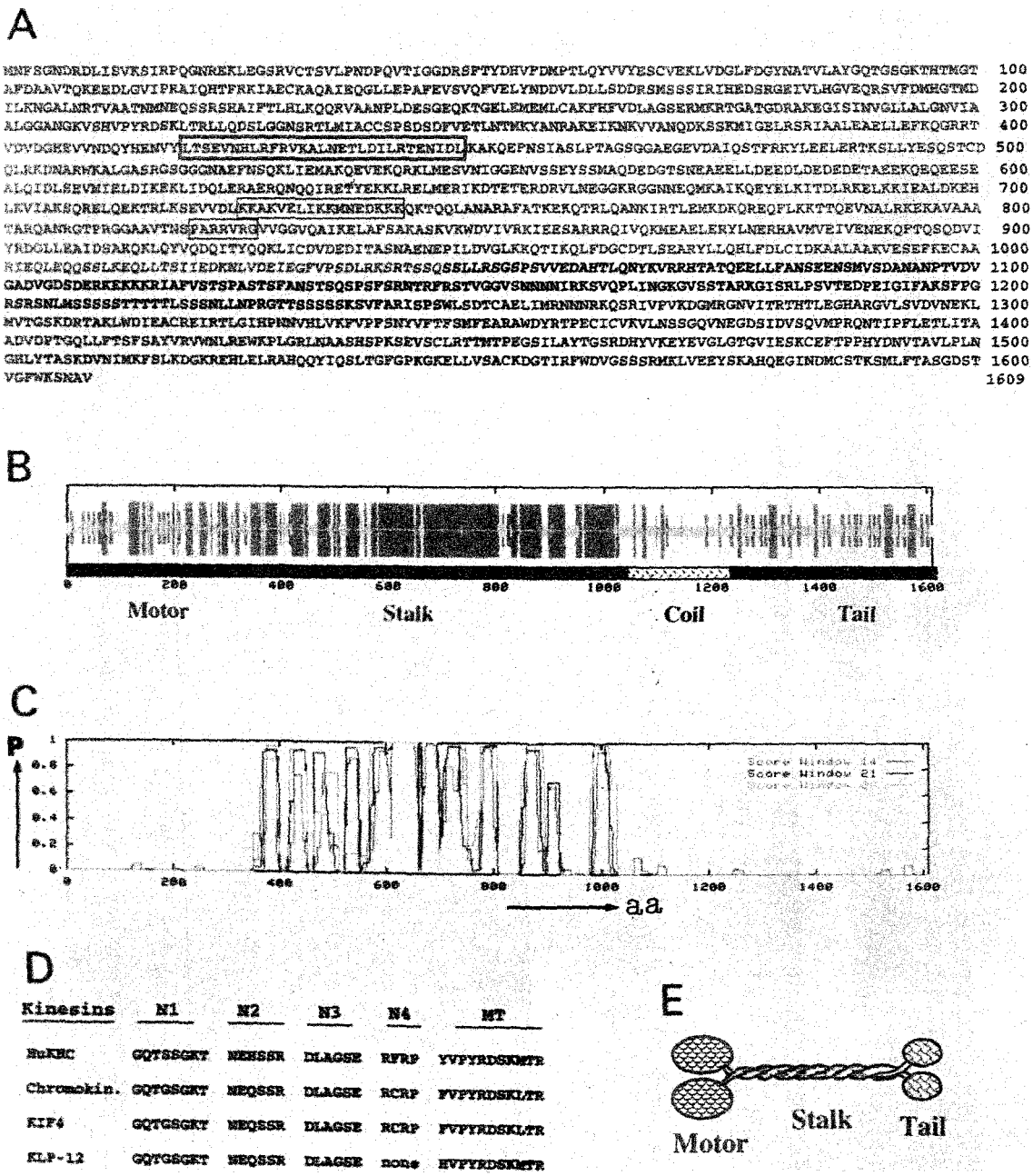

Fig. 2. Structure of the KLP-12 protein. **A.** Amino acid sequence of the KLP-12 protein of 1609 residues is shown by a single-letter amino acid code. The red colored amino acid residues (residues 1-376) represent the N-terminus motor domain of KLP-12. Similarly, green color, an alpha helical coiled coil stalk domain of residues from 377 to 1045; black color, a coil domain (residues 1046-1246); and blue color (residues1247-1609), the tail domain, located at the C-terminus of the protein. The double border lined amino acid residues represent two leucine zipper motifs (residues 419-440 and 426-447) and the single border lined residues represent the nuclear localization signals. **B.** Secondary structure prediction of KLP-12. Alpha helical (blue), beta strands (red) and coil (yellow) are plotted against the amino acid number using a computer program (Frishman and Argos, 1996). 'Motor', 'Stalk', Coil and 'Tail' represent the motor domain, stalk domain, and coil domain and tail domain of the KLP-12 protein respectively. **C.** Under the sequence is given the probability abbreviated to the first digit for windows of 14, 21 and 28 residues and the corresponding heptad repeat in the coil. Amino acid (**aa**) numbers were plotted along the X-axis and the **p**robability (**P**) was plotted along the Y-axis. **D.** Shows the ATP nucleotide binding pockets (N1, N2, N3, N4) and the microtubule (MT) binding site of KLP-12 and for comparison, human kinesin heavy chain, chicken Chromokinesin and mouse KIF4. **E.** A cartoon showing the Chromokinesin subfamily proteins. It depicts a globular motor (a dimer) domain located in the N-terminus (red color), an alpha helical coiled coil stalk domain in the middle (green color) and a globular tail domain in the C-terminus of the protein (blue color) (see review, Bloom and Endow, 1994).

Chromokineisn family.

RNA expression of *klp-12* using *in situ* hybridization In order to reveal the mRNA expression of *klp-12*, we performed the RNA *in situ* hybridization on embryos (Fig. 4A) and the dissected gonads (data not shown) from young adults using a *klp-12* specific cDNA probe. We found that the *klp-12* mRNA is expressed heavily throughout the embryos in the early stages up to 16 cell. But the signal is localized in the anterior nervous system in the head region in late embryos and is not seen in the early embryos (Fig. 4A). These observations suggest that *klp-12* is involved in the early development of cell division and in the nervous system in embryos.

In comparison, when the *klp-17* cDNA, encoding a C-terminus motor kinesin, was used as a probe, the hybridization signal was observed in the cell nucleus during the *C. elegans* embryogenesis (Fig. 4B) (Ali and Siddiqui, 2000). The nuclear expression of the *klp-17* appears as early as the one-cell stage embryo and it strongly continues through 2-cell and 4- cell stages. The hybridization signal remains fairly strong during the subsequent development, including the cell proliferation and morphogenesis. Several control experiments were conducted to ascertain the specificity of the *klp-12* hybridization signal. Removing the *klp-12* probe, and using only the Bluescript vector as a probe, showed no staining. As a negative control, we used a *dpy*-20 probe (Clark *et al.*, 1995) for RNA *in situ* hybridization and found no staining in embryos.

Double Stranded RNAi causes the dead embryos and polyploid cell To study the *in vivo* function of a given gene in *C. elegans*, the double-stranded RNA interference (dsRNAi) technique that disrupts the gene function, has proved a powerful tool (Fire *et al.*, 1998). The phenotype caused by the RNAi is generally very specific and reproducible. We used the RNAi to eliminate the expression of KLP-12 by injecting the corresponding dsRNA into the maternal germ line of a hermaphrodite (Fire *et al.*, 1998). The double-stranded RNA of *klp-12* was made as described in the methods section and was microinjected into the gonads of the *C. elegans* hermaphrodites. The embryos were observed using a Nomarski optics about 16-24 hours following the dsRNA injection, in order to study the effect of the dsRNA. We found that the *klp-12* RNAi resulted in arrested embryos at the one-cell stage (Fig. 5D-E) and rarely found in two cells or multiple cell stages. Light microscopic observations on the arrested embryos clearly showed defects in the early cell divisions, as the chromosomes appeared abnormally organized and the cytokinesis was blocked. Arrested embryos were stained with the DAPI nuclear dye in order to visualize the chromosomes. The DAPI staining showed that the chromosomes in the arrested embryos are polyploid (Fig. 5E) and the spindles appear disorganized (data not shown). About 500 animals (L4 larval and young adult stages) were injected to observe the effect of the *klp-12* dsRNAi. Among the injected animals, 450 animals (90%) show the effect of RNAi. Among the affected animals, 132 animals (30%) are lethal and 328 animals (70%)

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KLP-12        1  MNFSGNDRDLISVKS-IRPQGNREKLEGSRVCTSVLPNDPQVTIGGDRSFTYDHVFDMPT
Chromokin.    1  MVREEEKGIPVRVVR-CRPLVPKETSEGCQMCLSFVPGEPQVIVGSDKAFTYDYVFDPSV
KIF4          1  -MKEEVKGIPVRVALRCRPLVSKEIKEGCQTCLSFVPGEPQVVVGNDKSFTYDFVFDPST
                    .  .   : *    **   :*  **.: * *.:*.:*** :*.*::****.*** ..

KLP-12       60  LQYVVYESCVEKLVDGLFDGYNATVLAYGQTGSGKTHTMGTAFDAAVTQKEEDLGVIPRA
Chromokin.   60  EQEEVFNTAVAPLIRGIFKGYNATVLAYGQTGSGKTYSMGGTYTASQEHDP-SMGVIPRV
KIF4         60  EQEEVFNTAVAPLIKGVFKGYNATVLAYGQTGSGKTYSMGGAYTAEQEHDS-AIGVIPRV
                  *  *:::.*  *: *:*.******************::** :: *   :.   :*****.

KLP-12      120  IQHTFRKIAECKAQAIEQGLLEPAFEVSVQFVELYNDDVLDLLSDDRSMSSSIRIHEDSR
Chromokin.  119  IKLLFKEK--------EQ-RQDWEFVLKVSYLEIYNEDILDLLCSSRERSSQISIREDPK
KIF4        119  IQLLFKEIN-------KK--SDFEFTLKVSYLEIYNEEILDLLCSSREKATQINIREDPK
                 *:  *::         ::   :  * :.*.::*:**:::****...*. ::.* *:**.:

KLP-12      180  GEIVLHGVEQRSVFDMHGTMDILKNGALNRTVAATNMNEQSSRSHAIFTLHLKQQRVAAN
Chromokin.  170  EGIKIVGLTERNVASARDTVSCLEQGNNCRTVASTAMNSQSSRSHAIFTICIDQK--KKN
KIF4        170  EGIKIVGLTEKTVLVASDTVSCLEQGNNSRTVASTAMNSQSSRSHAIFTISIEQR--KKN
                   * : *: ::.*    .*:. *::*   ****:* **.**********: :.*:    *

KLP-12      240  PLDESGEQKTGELEMEMLCAKFHFVDLAGSERMKRTGATGDRAKEGISINVGLLALGNVI
Chromokin.  228  DKNSS------------FHSKLHLVDLAGSERQKKTKAEGDRLKEGININRGLLCLGNVI
KIF4        228  DKNSS------------FRSKLHLVDLAGSERQKKTKAEGDRLREGININRGLLCLGNVI
                   :.*            : :*:*:******** *:* * *** :***.** ***.*****

KLP-12      300  AALGGANGKVSHVPYRDSKLTRLLQDSLGGNSRTLMIACCSPSDSDFVETLNTMKYANRA
Chromokin.  276  SALGEENKKGGFVPYRDSKLTRLLQDSLGGNSHTLMIACVSPADSNLEETLNTLRYADRA
KIF4        276  SALG-DDKKGNFVPYRDSKLTRLLQDSLGGNSHTLMIACVSPADSNLEETLNTLRYADRA
                 :***  : * ..********************:****** **:**:: *****::**:**

KLP-12      360  KEIKNKVVANQDKSSKM
Chromokin.  336  RKIKNKPIVNVDPQAAE
KIF4        335  RKIKNKPIINIDPQAAE
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Fig. 3. Amino acid sequence homology among the Chromokinesin protein family. The motor domain of *C. elegans* KLP-12 shows a 49% sequence identity with the motor domains of both the chicken Chromokinesin and the mouse KIF4.

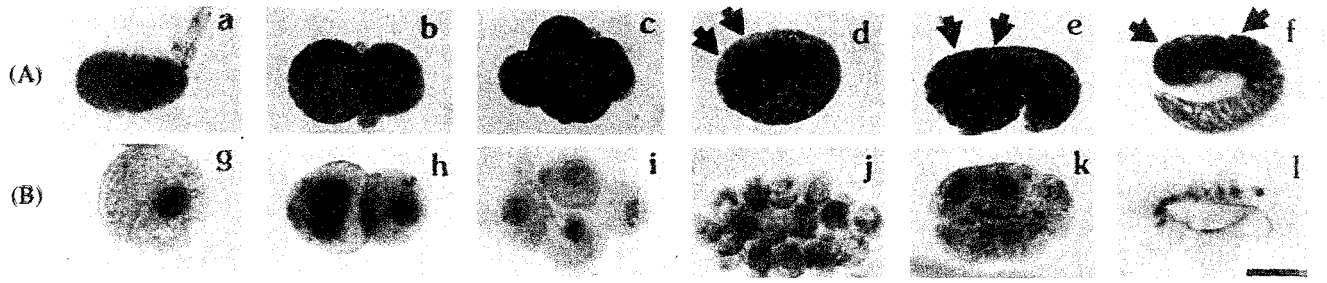

Fig. 4. Expression pattern of the *klp-12* gene. **A.** *Insitu* RNA hybridization pattern of *klp-12*. **a-c,** one-cell, two-cell and four-cell stages embryos respectively, show intense staining throughout the embryos during the development. **d-f,** multiple cells, comma cell and a larvae respectively, show intense staining in the anterior part of the embryos and larvae **B.** In comparison, *klp-17* (**g-l**) shows expression within the nucleus of the cell during the entire embryogenesis. Scale bar represents 10 micron.

show arrested embryos at the one-cell stage. We observed 470 one-cell arrested embryos. These results suggest that the *klp-12* gene has a critical role in the chromosomal movement during early embryonic and germ-line development.

Discussion

The Chromokinesin was first characterized in chicken. The protein contains both the conserved ATP and microtubule binding motor domain, as well as an atypical basic leucine-zipper DNA binding motif. The RNA *in situ* hybridization data revealed that the Chromokinesin mRNA is transcribed in mitotically active cells, but not in the post-mitotic cells (Wang and Adler, 1995). The immunocytochemical localization of Chromokinesin in the nucleus, and its primary association with chromosome arms, suggested that the Chromokinesin is directly involved in the chromosome locomotion during mitosis (Wang and Adler, 1995). Following the discovery of the chicken Chromokinesin, similar proteins were reported in different species. The Xklp1, a Chromokinesin of *Xenopus laevis*, was shown to play a role in the maintenance of spindle bipolarity and chromosome assembly at the metaphase plate (Vernos *et al.*, 1995). The mammalian orthologs of Chromokinesin were reported earlier in mouse as KIF4 and in human (Sekine, *et al.*, 1994; Yan and Wang, 1997). The mouse KIF4 is colocalized with membranous organelles in the mitotic spindle (Sekine *et al.*, 1994) and the human chromokinesin was shown to express at high levels in the retinoblastoma cells (Yan and Wang, 1997).

(1) In this paper we present data describing molecular cloning, structural analysis, and the embryonic expression of the *klp-12* gene in *C. elegans*. The *klp-12* encodes a 1609 residues long kinesin-like protein (Fig. 2A). Based on the structure, expression, and genetic analyses, several lines of evidence suggest that the KLP-12 is a member of the Chromokinesin/KIF4 subfamily. First, the amino terminus residues 1-376, "motor domain" of KLP-12, shares a 49% homology with the motor domain of the chicken Chromokinesin (Wang and Adler, 1995) and the mouse Chromokinesin ortholog KIF4 (Sekine *et al.*, 1994) (Fig. 3). However, the protein sequence homology is quite low (only 12%) in the non-motor region. This is also true for other members of the Chromokinesin subfamily. They share a significant sequence identity in the conserved motor domain, but display a considerable variation in the stalk and tail domains.

(2) The *C. elegans* KLP-12 also contains two leucine zipper motifs in the stalk domain that is similar to the chicken Chromokinesin (Fig. 2). The leucine zipper motif has been described in several DNA binding proteins. Further, it has been shown for the chicken Chromokinesin that it binds to DNA (Wang and Adler, 1995). The presence of two leucine zipper motifs in the *C. elegans* KLP-12 and the motor domain homology, suggest the conservation of essential structural features between KLP-12 and the chicken Chromokinesin. The structural similarities between KLP-12 and other members of this subfamily raises the question of whether the KLP-12 is expressed in mitotically dividing cells, and whether KLP-12 performs a similar *in vivo* function as other Chromokinesin motors?

(3) The expression pattern of *klp-12* mRNA, as deduced by *in situ* hybridization, reveals that the gene is expressed in mitotically dividing embryonic cells (Fig. 4). As shown in Figure 4, the *klp-12 in situ* hybridization signal is very intense in the early stages of the embryos. This would suggest a significant role of *klp-12* in the early development. The hybridization signal is observed in the rest of the embryonic development, but is somewhat lower in intensity as compared to the 1-cell, 2-cell and 4-cell stage embryos. These data suggest a continuous requirement for the *klp-12* transcription during the mitotic proliferation of embryonic cells.

We also found a strong signal on the Northern blot analysis when the polyA$^+$ containing mRNA, isolated from mixed stage worms (embryos, larvae, and adults), was blotted using a *klp-12* cDNA probe (Fig. 1B). This suggests a high level of *klp-12* transcription in the entire development. Similarly, the germ-line *in situ* hybridization pattern also suggests that the *klp-12* gene is actively transcribed in developing gonad (data not shown). Finally, we wanted to determine the effect of disrupting the KLP-12 function in *C. elegans* and compare this with the proposed biological role of Chromokinesin motors.

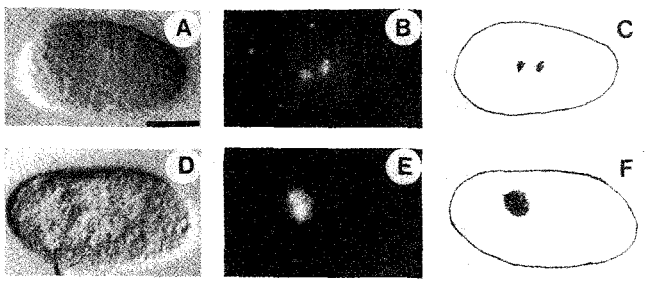

Fig 5. Double stranded RNA interference of *klp-12*. **A.** a wild-type one-cell embryo Nomarski micrographs. **B.** Same embryo as shown in panel A, stained with the DNA-staining dye DAPI, showing normal chromosomes in one-cell stage embryo. **C.** Cartoon depiction of the embryo shown in panel B. **D.** A *klp-12* dsRNA treated one-cell stage embryo that is arrested, Nomarski image. **E.** The embryo shown in panel D, stained with the DNA-staining dye DAPI, showing polyploid and mispositioned chromosomes. **F.** A Cartoon of embryo shown in panel E, highlighting the extra chromosomes, and a few abnormal chromosome, located in a abnormal position in embryo. Scale bar represents 12 micron.

(4) The dsRNA mediated RNA interference assay clearly shows that the absence of the *klp-12* function results in arrested cell divisions and the lethality of embryos (Fig. 5). Although the RNAi assay is limited by the fact that it does not provide a permanent mutant line, nevertheless the mutant phenotype, revealed by the gene disruption that is caused by the injection of dsRNA, provides a reproducible and reliable gene specific phenotype (Fire *et al.*, 1998). The predominant phenotype of the *klp-12* RNAi embryos is an arrest at the one-cell stage (Fig. 5), although embryos in two-cell or a multiple cell stage could also be observed when treated with *klp-12* dsRNA (data not shown).

In all of these cases, the arrested embryos show the accumulation of extra DNA, apparently caused by the failure of the chromosomes to segregate to the daughter cells. The chromosomal DNA in the arrested embryos, as visualized by staining with the nuclear dye DAPI, suggests that the arrested cells indeed possess extra DNA that was not properly segregated to the daughter cells (Fig. 5). These data point to the direct role of KLP-12 in chromosomal distribution. In our control experiments, *osm-3* that encodes another kinesin like protein in *C. elegans* (Shakir *et al.*, 1993; Tabish *et al.*, 1995), was used to perform the RNAi assay. No embryos were affected in the embryonic development and the chromosome distribution appeared normal. Thus, the RNAi phenotype, due to the *klp-12* dsRNA injection, is very specific and suggests that KLP-12 plays a role in chromosomal segregation. This data strongly indicates that the nematode KLP-12 is an ortholog of the chicken Chromokinesin that may function similar to the Chromokinesin in the proper segregation of chromosomes in the early embryonic development.

Based on the *klp-12* expression data and the RNAi assay (Fig. 4 and 5), we propose that KLP-12 may be required for normal chromosome assembly to an organized metaphase plate. We also suggest that the KLP-12 helps in the proper distribution of chromosomes to different poles. This is based on the observations of the arrested embryos lacking *klp-12* function due to the RNA interference. However, we ascribed no role to the KLP-12 during post-embryonic development, since we know little about the KLP-12 expression during larval and adult development.

Several related questions become obvious: How many different kinesin motors are involved in the process of cell division and chromosomal movement? Are there unique motors in terms of structure and function, or do they have a functional overlap? Is the amount of different kinesin motors important for the coordinated transport of cellular cargo? Data presented here suggests that although multiple kinesin motors are present in a simple animal, they appear to perform unique functions. The RNAi experiments clearly suggest that the inactivation of one kinesin motor cannot be easily substituted by another motor. Also, a large battery of kinesin motors are required in order to ensure proper spindle orientation and chromosome distribution during the early embryonic development.

With the availability of the *klp12* gene and its functional analysis, several questions can be asked. For example, what is the temporal and spatial pattern of expression of the KLP-12 protein? Are there other KLP-12 like kinesin proteins in *C. elegans* that may partially substitute for the absence of KLP-12? A number of lethal mutations have been mapped in several genes in the region of the *klp-12* gene on the linkage group IV (Fig. 1A). To identify the Chromokinesin mutant, we are rescuing several putative mutations, by germ-line transformation using the genomic *klp-12* DNA. Mutations in the *klp-12* gene will permit a systematic genetic analysis of the *klp-12 in vivo* function and allow an understanding of the upstream and downstream genetic control elements that govern the expression of the Chromokinesin gene. Our results provide a basis on which to explore the role of the chromokinesin subfamily in metazoan development.

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